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Effects of Rutin from *Toona sinensis* on the immune and physiological responses of white shrimp (*Litopenaeus vannamei*) under *Vibrio alginolyticus* challenge

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ABSTRACT

Rutin is a bioflavonoid with strong antioxidant activity. To investigate the regulatory roles of rutin in various functions in crustaceans, we examined physiological (haemolymph glucose, lactate, and lipid) and innate non-specific immune responses (total haemocyte count (THC), phenoloxidase activity (PO), respiratory bursts (release of superoxide anion, O_2^-) and superoxide dismutase (SOD) activity) to the pathogen *Vibrio alginolyticus* in white shrimp (*Litopenaeus vannamei*) that were individually injected with rutin extracted from *Toona sinensis* at 10, 20, or 50 $\mu g\,g^{-1}$. Results showed that PO activity and respiratory burst of *L. vannamei* increased obviously ($P < 0.05$) when injected with rutin at a dose of 20 and 50 $\mu g\,g^{-1}$ after 12 and 24 h, respectively. Both the THC and SOD activities of experimental and control groups revealed no significant difference at all doses. *L. vannamei* injected with rutin at either dose maintained lower glucose, lactate, and lipid levels in response to *V. alginolyticus* challenge after 12–36, 24–48, and 24–60 h, respectively. The survival rate of *L. vannamei* that received rutin at either dose was significantly higher than that received saline after 48–72 h. It was, therefore, concluded that the immune ability and resistance against *V. alginolyticus* infection of *L. vannamei* receiving rutin at $\geq$10 $\mu g\,g^{-1}$ increased.

1. Introduction

Many plant-derived compounds are known to have non-specific immune-stimulatory properties in animals, of which more than a dozen have been evaluated in fish and shrimp [1,2]. Even though glucan and other immunostimulants have positive effects on fish and prawn [3–5], some disadvantages have been found with the application of these natural immunostimulants like being intolerant to heat and indigestible. Hence, it is advisable to continue searching for alternative immunostimulant products from plants.

Toona sinensis Roem. (Meliaceae) is distributed in southern Taiwan. The Hakka society diet popularly consisted of this plant. The leaves and stems of this plant have been used for the treatments of enteritis, dysentery, and itch in oriental medicine [6]. Previous phytochemical work on *Toona* species had led to the isolation of triterpenes and phenolic compounds [7]. To further search for immune protective agents from *T. sinensis*, rutin was chosen to help maintain a healthy immune system. Rutin, or quercetin-3-rutinoside, is one of the most commonly found flavonol glycosides identified as vitamin P together with quercetin and hesperidin and is widely present in many plants, especially the buckwheat plant [8]. Rutin has been reported to have clinically relevant functions, including antioxidant, antihypertensive, anti-inflammatory, and antihemorrhagic activities, strengthening of the capillaries and the regulation of capillary permeability, and stabilization of platelets [9,10]. However, little efforts have been made to determine the impacts of rutin application against pathogen infection in shrimp.

The white shrimp, *Litopenaeus vannamei*, which is naturally distributed along the Pacific coast of Central and South America, was introduced to Taiwan in 1985, and since then, techniques for broodstock husbandry, manipulated spawning, and hatchery technologies for mass seed production have been successfully developed. It has soon become the most important cultured species in Taiwan [11]. In consequence, aquaculture production of *L. vannamei* in Taiwan reached 10,432 metric tons (mt) in 2006, with a unit production of 6 mt ha^{-1} [12]. However, in recent years, the shrimp culture industry has often suffered from losses attributed to outbreaks of infectious viral and bacterial diseases. Thus, immunity enhancement of cultivars for improved resistance and survival in fluctuating environments and with pathogen infection, and thereby

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preventing or reducing disease outbreaks are crucial for continued and sustained growth of the shrimp culture industry.

All forms of stress activate responses through diverse physiological processes, and among them, energy metabolism is of prime importance for physiological compensation by organisms [13,14]. Stress can also suppress the defense system to such an extent that susceptibility to disease is increased [15]. Since aquatic organisms are constantly subjected to environmental fluctuations and are under challenges from potential pathogens in the aquatic environment, reciprocal changes in the physiological and immune processes are anticipated.

Previous research indicated that the total haemocyte count (THC), differential haemocyte count (DHC), phenoloxidase (PO) activity, respiratory bursts (O_2^-), and superoxide dismutase (SOD) activity are commonly used as functional parameters for evaluating the immune potential [16]. Furthermore, these parameters have been reported in the white shrimp *L. vannamei* [17–19]. The purpose of this study was to determine whether penaeid shrimp receiving rutin show increased immunity and enhanced resistance to *Vibrio* infection. Accordingly, several physiological parameters such as haemolymph glucose, lactate and lipid, and several immune parameters including the THC, PO activity, O_2^-, and SOD activity of *L. vannamei* were examined. The susceptibility of the shrimp to *Vibrio alginolyticus* when they were injected with rutin was also monitored.

2. Materials and methods

2.1. Culture of V. alginolyticus

The bacterium, *V. alginolyticus* (CH003), was isolated from diseased *L. vannamei* [20]. A known pathogenic strain of *V. alginolyticus*, 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, Michigan) for 24 h at 28 °C and then transferred to 10 ml of a 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 1×10^7 colony-forming units (cfu) ml^{-1} for the susceptibility test.

2.2. Rutin product

The natural product of rutin was isolated from *T. sinensis* [21]. The leaves of *T. sinensis* were collected from Fooyin University, Ta-Liao, Kaohsiung County, Taiwan, in January 2003. The leaves of *T. sinensis* (6 kg) were extracted with MeOH (10 L × 6) at room temperature for 24–48 h. The combined MeOH extracts were evaporated under reduced pressure to yield dark-brown syrup (266.7 g). The MeOH extracts (120.0 g) was chromatographed over silica gel (*n*-hexane/EtOAc/MeOH) to obtain 35 fractions. The fraction 23, eluted with EtOAc/MeOH (8:1), was further separated and purified by capillary electrophoresis using silica gel column chromatography to yield rutin (1290 mg). Rutin was dissolved in 75% EtOH to concentrations of 5, 10, and 25 mg ml^{-1} before the injection.

2.3. L. vannamei

L. vannamei shrimps averaging 11.69 ± 0.8 g (mean ± SD) were obtained from King Car Industrial Co., Ltd. (Ilan, Taiwan) and acclimated in the laboratory for 2 weeks before experiments started. Only shrimps at the intermolt stage were used for the study. The molt stage was determined by morphological examination of uropods in which partial retraction of the epidermis could be distinguished [22]. For the susceptibility experiment, the test and control groups were comprised of 10 shrimps each in triplicate. For monitoring the physiological and immunological response parameters: haemolymph glucose, lactate, triglyceride level, total haemocyte count, phenoloxidase, respiratory bursts and superoxide dismutase activity, the test and control groups composed of eight shrimps for each time interval. In all tests, shrimps 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‰.

2.4. Effect of rutin on the susceptibility of L. vannamei to V. alginolyticus

L. vannamei shrimps were individually injected into the ventral sinus of the cephalothorax with 5, 10 and 25 mg ml^{-1} rutin solution (around 20 μl) to achieve doses of 10, 20 and 50 μg g^{-1} body weight, respectively, on day 1. The shrimps injected with 75% EtOH (20 μl) served as the saline group. 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. Shrimps that received no rutin and thenreceived *V. alginolyticus* at 2×10^5 cfu $shrimp^{-1}$ served as the challenged control, while those received no rutin, and then received saline (20 μl) served as the unchallenged control (Table 1). Experimental and control shrimps, 30 in each treatment, were maintained in 60 L glass aquaria containing 40 L of water at 32‰. Water was renewed daily, and the experiment lasted 72 h.

2.5. Immune parameters of L. vannamei to V. alginolyticus

Haemolymph samples were collected at varying time intervals of 0, 12, 24, 36, 48, 60, and 72 h. Haemolymph (100 μl) was withdrawn from the ventral sinus of each shrimp into a 1-ml sterile syringe (25 gauge) containing 0.9 ml of an anticoagulant solution

Table 1
Effect of rutin extracted from *Toona sinensis* on the survival of white shrimp, *Litopenaeus vannamei*, challenged with *Vibrio alginolyticus*

Bacterial (cfu $shrimp^{-1}$)	Rutin (μg g^{-1})	No. of shrimps	Survival rate (%), and number of shrimps surviving (in each group) at various times after challenge (h)					
			12	24	36	48	60	72
Saline	Control	30	100	100	100	100	100	100
2×10^5	Control	30	93.3 ± 3.3^a	86.7 ± 8.9^b	73.3 ± 5^b	66.7 ± 3.3^b	60.0 ± 5.8^b	46.7 ± 7.7^c
2×10^5	Saline	30	93.3 ± 3.3^a	86.7 ± 8.9^b	73.3 ± 5^b	66.7 ± 6.6^b	63.3 ± 3.3^b	50.0 ± 5.8^c
2×10^5	10	30	100 ± 0^a	96.7 ± 3.3^a	93.3 ± 3.3^a	$76.7 \pm 3.5^{a,b}$	$76.7 \pm 3.5^{a,b}$	66.7 ± 6.6^b
2×10^5	20	30	100 ± 0^a	100 ± 0^a	93.3 ± 3.3^a	83.3 ± 6.4^a	83.3 ± 6.4^a	76.7 ± 3.5^a
2×10^5	50	30	100 ± 0^a	100 ± 0^a	93.3 ± 3.3^a	83.3 ± 6.4^a	83.3 ± 6.4^a	76.7 ± 3.5^a

Data in the same column with different letters significantly differ ($P < 0.05$) among different treatments.
Values are the mean ± SE ($n = 30$ shrimp in each treatment).
cfu, colony-forming unit.