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# Investigation of *Scutellaria radix* on Phenoloxidase and Prophenoloxidase of Giant Freshwater Prawn, *Macrobrachium rosenbergii*, via Reverse-gavage Feeding

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**KEYWORDS**

*Scutellaria radix*
*Macrobrachium rosenbergii*
Immune response
Reverse-gavage feeding

**Abstract** *Scutellaria radix* is one of the ancientest approaches to treat diseases. The aim of this study was to investigate the effect of *Scutellaria radix* on the immune responses of *Macrobrachium rosenbergii* by reverse-gavage feeding and their protection against *Lactococcus garvieae*. In brief, *M. rosenbergii* was injected with *S. radix* water extract at 1, 5 and 10 µg/µl via reverse-gavage feeding (RGF). Phenoloxidase (PO) activity of hemocytes and prophenoloxidase (proPO) gene of hepatopancreas were assessed to determine *M. rosenbergii* immunity. The results revealed that the concentration of *S. radix* extract at 5 µg/µl enhanced significantly the phenoloxidase of *M. rosenbergii* correlated with the control group. For pro PO were up-regulated by all concentration in a time-dependent manner. The highest percentage of survival rates was observed at 5 µg/µl of *S. radix* water extract after challenged with *L. garvieae*. The present discoveries suggest that *S. radix* water extract at the concentration of 5 µg/µl may raise the immune responses and gene expressions in *M. rosenbergii* using RGF.

## Introduction

*Macrobrachium rosenbergii* is one of the most common, profitable prawn and has been found in more than 40 countries (New, 2012). However, the intensive production of *M. rosenbergii* has generated a problem of diseases (Chen *et al*., 2001). The major disease-causing agents of giant freshwater prawns are viruses, bacteria, protozoa, and fungi/yeast (Suanyuk *et al*., 2014). Recent studies revealed that *L. garvieae* has led to huge losses of giant freshwater prawns in Taiwan facilitated by inferior environmental parameters (e.g. temperature and pH) in the hot season (Halim *et al*., 2017).

Antibiotics are still used to deal with bacterial diseases (Serrano, 2005). However, it is decided that overuse of antibiotics can diminish the ability of antibiotics (Sorum *et al*., 2006) and increase environmental hazards (Guo *et al*., 2011). Another issue is residual antibiotics in commercialized fish, shrimp, and shellfish stocks (Lakshmi *et al*., 2013). Based on a study of Bermúdez-Almada and Espinosa-Plascencia (2012), oxytetracycline, the most globally administered in commercial aquaculture, was concentrated in the tissue and hepatopancreas of shrimp when it was applied to deal with the bacterial infections in the shrimp production. Then, the development of non-antibiotic approaches is needed for health control in shrimp culture (Guo *et al*., 2011).

It is clear that herbal medicine is the most ancientest approach worldwide application to treat diseases (Disch *et al*., 2017). The *S. radix* is extensively applied as a medicinal plant for remedy of diverse diseases and food supplement (Ohtsutki *et al*., 2009, Zhou *et al*., 2016, and

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Króliczewska *et al*., 2017). *S. radix* contains high concentrations of flavones and flavone glycosides (baicalein (Yue *et al*., 2012), wogonin (Huang *et al*., 2003) and their glycosides), which are also the main active compounds. Flavones and flavone glycosides in *S. radix* are used effectively for improving immunity (Króliczewska *et al*., 2017, and Cho *et al*., 2013), anti-inflammatory (Yoon *et al*., 2009), antiviral (Zhong *et all*., 2013), anticancer (Sato *et al*., 2013), antibacterial (Kim and Nam, 2017) and other biological activities (Jung *et al*., 2012).

Therefore, the aim of this study was to examine the immune responses of *M. rosenbergii* and its tolerance to *L. garvieae* post-RGF of *S. radix* extract. Immune parameters, phenoloxidase (PO), and the expressions of immune related-genes, such as prophenoloxidase (proPO) were examined.

## Materials and methods

*Prawn and plant extraction*

*M. resenbergii* (20.0 ± 2.0 g) were obtained from a commercial farm in Pingtung (Taiwan), and reared in National Pingtung University of Science and Technology. Prawns (25 prawns/tank) were acclimatized at 25°C for two weeks before conducting experiment and fed with commercial feed at 5% of shrimp body weight per day. Water quality was controlled by the mechanical circulating system to keep the environment in good condition.

*S. radix* extraction was followed by a study of Zhao *et al.* (2016) with some modifications. Briefly, *S. radix* ground to powder, then 10 gram of *S. radix* were added to 150 mL of double-distilled water ($ddH_2O$) and boiled in a pot at 95°C for 15 minutes. After the solution was cooled down, it was transferred and divided carefully into falcon tubes for centrifugation. Centrifugation was conducted at 4°C, 1000 g for 10 minutes. The supernatant was then transferred to 500 mL serum bottle, autoclaved, and stored at -20°C. The clear supernatant of *S. radix* water extract was evaporated using freeze dryer. Then, the powder was stored at 4°C until use.

*Effect of S. radix extract on the immune response*

First, the powder was dissolved in PBS solution at different concentrations of 0, 1, 5, and 10 µg/µl (T0, T1, T2, and T3, respectively). The solution was then mixed with red food dye (Uchi Co., Ltd) in a 1:30 v/v (red dye: PBS) for the observation of solutions inside hindgut, midgut, and hepatopancreas (HP). After fasted for 24 hours, the prawns were injected from their anuses with the mixture by pipettement. Ten microlitres of solutions were inserted using a micropipette of 0.1–10 µl during the injection.

Hemolymphs and HPs from total ninety-six prawns (20.0 ± 2.0 g) injected with the extract were collected at 3, 6, 12, and 24 hours post-RGF. While the hemolymphs were employed for assessing the immune responses, the HPs were needed for determining the expressions of immune-related genes. For hemolymph, 600 µl were obtained carefully from the ventral sinus of the shrimp using sterile 1 mL syringes. One hundred microlitres of hemolymph were transferred to 1.5 mL sterile eppendorf tube containing 900 µL anticoagulant (0.8 g sodium citrate, 0.34 g EDTA, and 10 µl Tween 80 in 100 ml of distilled water, at pH 7.45). For HPs, they were collected separately and immediately suspended in RNA later (QIAGEN) and kept at −80°C until use.

Based on methods from Hernandes *et al.* (1996) and Wu *et al.* (2015), PO activity was spectrophotometrically calculated by reading the structure of dopachrome generated from L-dihydroxyphenylalanine (L-DOPA) with some modifications. The optical density (OD) was measured at 450 nm using ELISA reader and expressed as dopachrome formation per 50 µl of hemolymph.

*Effect of S. radix extract on the immune-related genes*

Total RNA was extracted from HPs using Trizol® reagent (Invitro, USA) conforming to the

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company's guidance. The RNA samples were arrested in 30µl DECP-treated water and stored at -80°C until use. Total RNA was quantified and qualified using Biospectrometer (Germany), with the OD260/OD280 rate of 1.8-2.0. First strand cDNA was then extracted from 2 µg of RNA employing M-MuLV reverse transcriptase (Lucigen) conforming to the company's instructions. The cDNA was finally kept at −20°C.

Real-time PCR was operated employing an ABI StepOnePlus™ Real-time PCR system (Applied Biosystem, USA). The amplification mixed 5 µl of SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Bio Inc), 0.2 µl of ROX reference dye (50x), 0.2 µl of each primer, 1 µl of cDNA template, and 3.4 µl of DEPC treated water. The real-time PCR was observed keeping the condition as 95°C for 1 minute followed by 40 cycles of 95°C for the 15s, and 60°C for 1 minute.

**Table 1. RT-qPCR primers of immune-related genes**

| Target genes | Genebank | Primer | Sequence (5'-3') |
|---|---|---|---|
| proPO (Prophenoloxidase) | AY947400 | F | ACACTGAAGGACATAAGGCGAGAT |
| | | R | AGTAGAGTTCCAAGTCGGAGATGCT |
| Mrß-actin, | AY651918.1 | F | CCCGACGGTCACTTGTTC |
| | | R | CGTGGATGCCGCAGGATT |

*Challenge and evaluation of mortality rate*

The prawn-pathogenic strain of *L. garvieae* was kindly prepared by Chen *et al*. (2001) and used in this study. *L. garvieae* was cultured on a tryptic soy agar (TSA) at 28°C for 24 hours before being moved on to 15 ml of tryptic soy broth (TSB). Thereafter, the bacteria were incubated at 28°C for 24 hours, then centrifuged at 3000 g for 15 minutes at 4°C. After separating the supernatants, the pellets were resuspended in PBS (0.85% NaCl) at a concentration of $6 \times 10^6$ CFU $ml^{-1}$ for a challenge.

The challenge experiment was run at 24 hours post-RGF. 150 prawns were vaccinated with 20 µl of *L. garvieae* ($6 \times 10^6$ CFU/prawn) into the ventral sinus. Mortality of prawns was observed during 96 hours post-challenge.

*Statistical Analysis*

ANOVA (One-way analysis of variance) was used to calculate variations among groups. The Tukey test was employed to investigate significant differences among treatments using SPSS. Data were presented as the mean ±SD, $p<0.05$ was considered significant.

**Results and discussions**

Hemocytes, one of the most important defense mechanisms, are the mediators of cellular immunity and play a crucial role against pathogens (Estrada *et al*., 2006). As in hemocyte, HP is also one of the signal organs to determine the shrimp status condition (Parrinello *et al*., 2015). Moreover, HP, or referred to midgut gland, is also considered as an essential location for the expression of the immune-related gene in shrimp (Dall, 1967). On the other hand, pharmacological investigations have confirmed that the *S. radix* shows several biological activities such as antitumor, hepatoprotection, antibacterial, antiviral and other activity effects (Zhao *et al*., 2016). In our study, it is revealed *S. radix* extract can modulate immunity of *M. rosenbergii*. It enhanced the innate immune responses, the expressions of immune related-genes in prawns, and decrease in mortality rate of prawn post-challenge with *L. garviceae*.

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*Effect of S. radix extract on phenoloxidase*

PO plays an important role in melanization, which is known as the innate immune cascade to encapsulate and eliminate pathogens (Amparyup *et al*., 2013, Cerenius *et al*., 2008). In the present study, *S. radix* extract can enhance rapidly the PO activity at different concentrations. During 24 hours, the highest expression of PO was showed at T1 at 3 hours post-RGF and followed by T2. At 6 hours post-RGF, while T2 and T3 showed significant increases, T1 showed no significant difference from the control group. Decreasing PO activity happened post-RGF 12 hours and all treatments had a similar amount of PO compared with the control group ($p < 0.05$) at 24 hours post-RGF, except T2. It is obvious that T2 (5µg/µl) displayed the greatest rate of the PO at all time point. The similar result, the increase in PO of *L. vannamei* was observed at the concentrations of 4 and 8 µg/g at 12 hour post-injection using *Gynura bicolor* extract (Wu *et al*., 2015). In addition, enhanced PO was also confirmed by some herbal medication such as leaves of noni, *Morinda citrifolia* (Halim *et al*., 2017) and ginger extract (Chang *et al*., 2012), on *M. rosenbergii.* It is figured out that increasing of PO has been connected with the raised resistance to the infection (Brookman *et al*., 1989, Soderhall and Cerenius, 1998, Sugumaran, 2002, Shelby and Popham, 2006) and a decrease of PO activity revealed the depression of the immune system (Yeh *et al*., 2006). These discoveries report that *S. radix* could be employed to enhance *M. rosenbergii* PO using RGF.

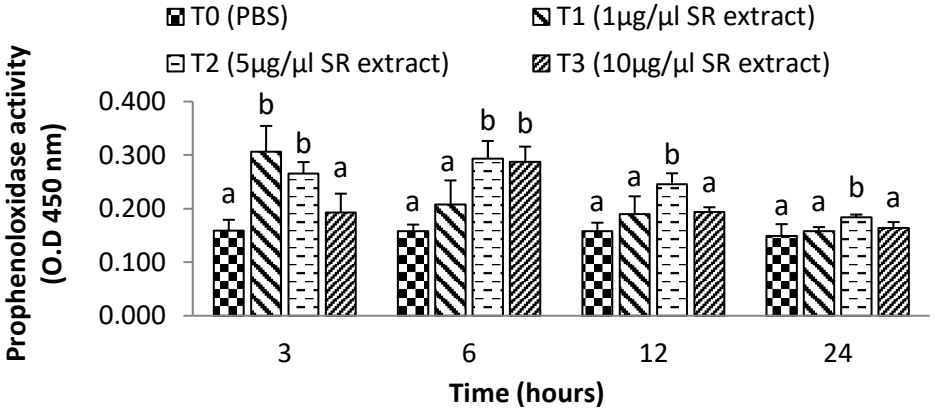


**Figure 1.** Phenoloxidase activities of *M. rosenbergii* post-RGF with *S. radix* water extract for 24 hours. Data (mean ± SD) with different letters are significantly different ($p$<0.05) among treatment.

*Effect of S. radix extract on the expressions of prophenoloxidase gene*

The proPO activating system is believed to be an essential innate defense in invertebrate immunity (Charoensapsri *et al*., 2011). Activation of proPO is showed to generate cytotoxic products, melanin production (melanization) and encapsulation of pathogen (Cerenius *et al*., 2008). The shrimp proPO could be synthesized in the hemolymph, gill, stomach, HP, and intestine (Pang *et al*., 2014, Charoensapsri *et al*., 2009, Rao and Anjaneyulu, 2008). Identification of proPO in various shrimp tissues showed that proPO is used for several functions, including growth encouragement or larval development (Charoensapsri *et al*., 2011), molting control (Rao and Anjaneyulu, 2008) and playing a central role in killing and eliminating of invading pathogens (Tassanakajon *et al*., 2018). Although proPO gene expression is the weakest expression in the shrimp HP in normal condition (Yeh *et al*., 2009), HP has been known as a huge gland essential for the expression of the immune-related gene in shrimp (Felgenhauer, 1992 and Dall, 1967).

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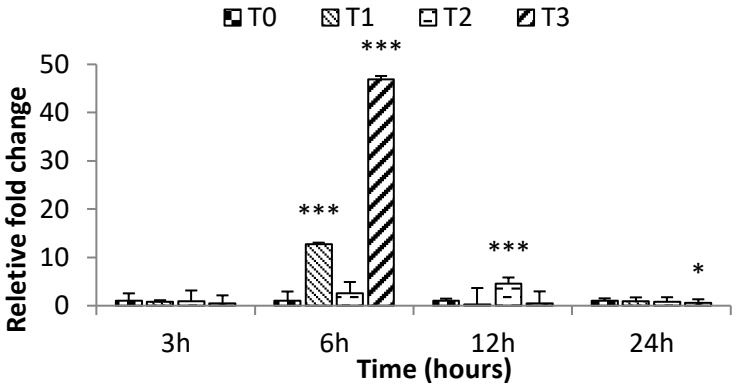


**Figure 2.** Expression of proPO in *M. rosenbergii* hepatopancreas post-RGF

In this investigation, the expression of proPO fluctuated during 24 hours. While T1 and T3 reached their peaks at 6 hours post-RGF (12.8- and 46.8-fold, respectively) and thus a decay pattern over time, T1 showed a peak at 12 hours (4.5-fold). Many investigations revealed that increase in proPO in the HP was created by bacterial and viral disease, laminarin, LPS, and poly I:C (Taju *et al*., 2015, Pizabo *et al*., 2014, Du *et al*., 2013, Ji *et al*., 2009), but no assessment of herbal medication on proPO expression in HP. We figure out that the rise of proPO in HP was triggered by phagocytes, which are identified hyaline (Thornqvist *et al*., 1994) and semigranular cells (Kakoolaki *et al*., 2010). According to Rőszer (2014), HP is strong in both motile phagocytes of hemolymph and fixed phagocyte (Johnson, 1987) which is an induction of melanization in the HP. In our situation, *S. radix* might stimulate *M. rosenbergii* phagocytes of hemolymph which is passing through HP. It was shown by *Musa acuminate* (Rattanavichai and Cheng, 2015), *Morinda citrifolia* (Halim *et al*., 2017), and *Solanum nigrum* (Harikrishnan *et al*., 2011). Both semigranulocyte (Kakoolaki *et al*., 2010) and hyaline cells (Supamattaya *et al*., 2003) show significant roles in release and storage of the proPO system (Johansson and Soderhall, 1985). Indeed, *P. monodon* phagocytes have been figured out to concentrate antigens in the HP as a warning to trigger an immune response (Alday-Sanz *et al*., 2002). Based on our results, it might be determined that *S. radix* extract enhances proPO in the *M. rosenbergii* HP by activation of phagocytes cell.

*Mortality rate of M. rosenbergii post-challenge with L. garvieae*

The mortality rate of *M. rosenbergii* post-challenge with *L. garvieae* was presented in Table 2. It is noticeable that the mortality rate of all treatments was dropped correlated with the control group. The smallest percentage was discovered in T2 (20%), accompanied by T3 and T1 (23.33 and 33.33%, respectively). Based on this result, the *S. radix* water extract may increase *M. rosenbergii* protection against *L. garvieae*.

**Table 2. The mortality of *M. rosenbergii* post- challenge with *L. garvieae***

| Challenge dose (cfu/ prawn) | Treatment | No. of prawns | Mortality (%) at different times (hour) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 6 | 12 | 24 | 48 | 72 | 96 |
| Saline | Negative control | 30 | 0 | 0 | 0 | 0 | 0 | 0 |
| $6 \times 10^6$ | T0 | 30 | 0 | 30 | 50 | 60 | 66.7 | 76.7 |
| $6 \times 10^6$ | T1 | 30 | 0 | 10 | 16.7 | 30 | 33.3 | 33.3 |
| $6 \times 10^6$ | T2 | 30 | 0 | 0 | 6.7 | 13.3 | 13.3 | 20 |
| $6 \times 10^6$ | T3 | 30 | 0 | 3.3 | 13.3 | 16.7 | 16.7 | 23.3 |

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A comparable effect was also studied in *Eichhornia crassipes* leaves that their extraction was demonstrated to decrease the mortality rate of *M. rosenbergii* post-challenge with *L. garvaeae* (47.8%) (Chang *et al*., 2013). On the other hand, *S. radix* was also decreased the mortality rate of Olive Flounder (*Paralichthys olivaceus*) tested with *E. tarda* (Cho *et al*., 2013), indicating that *S. radix* can decrease the mortality rate of diverse aquatic animals against bacterial diseases.

**Conclusions and suggestions**

*S. radix* water extract decreased the mortality rate of prawns post challenged with *L. garvieae* and also increased their PO and proPO expression. Up-regulation of the proPO gene in HP could not be raised precisely by *S. radix* water extract. Increasing of immune-related genes in HP trigged by phagocyte cells. In addition, this investigation revealed that the concentration of 5 µg/µl can be applied to increasing giant freshwater prawn immunity.

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