Reverse-gavage feeding as a novel administration to investigate the immunomodulatory effects of plant-based medicines on the immunity of giant freshwater prawn, *Macrobrachium rosenbergii*: A case study of *Scutellaria radix* water extract

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Abstract

The application of herbal medicine is one of the ancientest worldwide approaches to treating diseases. This study aimed to examine the effect of *Scutellaria radix* on the immune responses of *Macrobrachium rosenbergii* by reverse-gavage feeding and their resistance against *Lactococcus garvieae*. In brief, *M. rosenbergii* was administered with *S. radix* water extract at 1, 5, and 10 µg/µl using reverse-gavage feeding (RGF). Total hemocyte count (THC), phenoloxidase (PO) activity, respiratory bursts (RBs) activity, transglutaminase (TG) activity, and lysozyme activity of hemocytes were evaluated to discover the immunological responses of prawn post-RGF. The results showed that the concentration of *S. radix* extract at 5 µg $µl^{-1}$ increased the immune responses of *M. rosenbergii* compared with the control group. In the case of the gene, expressions were observed from the hepatopancreas and midgut, in which proxinectin showed no significant difference, LGBP and proPO were expressed by all concentrations in a time-dependent manner. The present findings suggest that *S. radix* water extract at 5 µg $µl^{-1}$ may enhance the immune responses and gene expressions in *M. rosenbergii* using RGF.

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, is a commercially important species throughout South-East Asia (Lalrinsanga et al 2014; Nguyen et al 2019). However, the rapid development of industry has promoted the incidence of infectious diseases on a large scale (Chen et al 2001; Hsu et al 2005; Suanyuk and Dangwetngam 2014). *Lactococcus garvieae* infections have resulted in significant production losses and substantial financial losses (Bilen et al 2019). Based on Baños et al (2019) study, *L. garvieae* infection led to 100% mortality of Trout for 15 days. Another specific study also decreased the survival rate of *M. rosenbergii* after 144 hours of exposure, accounting for 16.7%. Those problems make farmers apply antibiotics to combat the disease, generating severe consequences. Antibiotic consumption can result in antibiotic-resistant bacteria, antibiotic residues in fish, and public health concerns (Cabello 2006; Liu et al 2017). Antibiotics are extensively condemned for their excessive and widespread use, which has resulted in their accumulation and contamination in the aquatic environment (Lian et al 2016). As a result, it needs a solution to solve antibiotic problems, such as plant-based medicines that have been recently used as therapeutic alternatives (Disch et al 2017; Valladão et al 2015).

In aquaculture, medicinal herbs have been reported to promote growth, improve immunity, and possess antimicrobial properties (Direkbusarakom et al 1998; Jian and Wu 2004; Sivaram et al 2004; Yahaya et al 2015). In this case, *Scutellaria radix* is one of the family Lamiaceae used in traditional medicine. It is rich in flavones and flavone glycosides which have positive effects on animal health, such as immunostimulation (Cho et al 2013; Króliczewska et al 2017), anti-inflammation (Yoon et al 2009), anticancer (Sato et al 2013), and anti-virus (Zhong et al 2013). Recently, several studies were conducted to evaluate the effects of supplemental *S. radix* on the performance of animals, including olive flounder (Cho et al 2013), tilapia (Yin et al 2006), and chicken (Króliczewska et al 2017). However, to the best of our knowledge, no study has considered the effects of *S. radix* on farmed prawns.

Moreover, injection, immersion, and oral administrations are the most common delivery routes of plant extracts in aquaculture (Reverter et al 2014). Each method has advantages and disadvantages that have been reviewed previously (Assefa and Abunna 2018; Galindo-Villegas and Hosokawa 2004). On the other hand, reverse gavage was reported as a practical challenge method while minimizing discomfort and trauma to shrimp (Tran et al 2013). The result also suggested that reverse gavage can characterize clinical signs associated with dosage. Therefore, reverse gavage feeding (RGF) is a potential method for a fast interpretation of the systemic effect of plant-based medicines.

Hence, the present study aimed to investigate the immunostimulant activity of *S. radix* in prawns after reverse gavage feeding (RGF).

2. Materials And Methods

2.1. Prawn and plant extraction

M. resenbergii (20.0 ± 2.0 g) were acclimatized at 25^{o}C with continuous aeration and fed with commercial feed at 5% of shrimp body weight per day for two weeks before treatment.

S. radix extraction was prepared following a method modified by Zhao et al. (Zhao et al 2016). Briefly, *S. radix* was ground into powder, then 10 g of *S. radix* were added to 150 mL of double-distilled water (ddH_2O) and heated at 95^{o}C for 15 min, followed by centrifugation at 4^{o}C, 1000 g for 10 minutes. The supernatant of *S. radix* water extract was collected and evaporated using a freeze dryer (EYELA FDU-1100). Finally, the powder was kept at 4^{o}C until use.

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

The powder was dissolved in PBS at 0, 1, 5, and 10 µg/µl (T0, T1, T2, and T3, respectively) and then mixed with red food dye (Uchi Co., Ltd) in a 1:30 v/v (red dye: PBS). After fasting for 24 hours, the prawns were reversely gavage fed with 10 µl of the mixture.

Hemolymphs, mid-guts (MGs), and Hepatopancreas (HPs) were collected at 3, 6, 12, and 24 hours post-RGF. The hemolymphs were used to evaluate the immune responses, and the MGs and HPs were used to analyze the expressions of immune-related genes (section 2.3). One hundred microlitres of hemolymph were transferred to a 1.5 mL eppendorf tube containing 900 µL anticoagulant (0.34 g EDTA, 0.8 g sodium citrate, and 10 µl Tween 80 in 100 ml of distilled water, at pH 7.45 with the osmolality adjusted to 490 mOsm kg^{-1} with NaCl). MGs and HPs were collected separately and immediately immersed in RNAlater (QIAGEN) and stored at − 80 ^{o}C until use.

The respiratory burst (RBs) was measured with a modified method from Song and Hsieh (Song and Hsieh 1994) using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) production. RBs was expressed as the amount of NBT reduction in 100 µl of hemolymph using OD at 620 nm.

Based on the modified colorimetric hydroxamate procedure, TG activity was directly measured from hemolymph at 525 nm (Gorman and Folk 1980). Briefly, 50 µL of hemolymph was incubated with 150 µL of Tris-acetate buffer, 12.5 µL of Hydroxylamine hydrochloride 2M, and 37.5 µL of Z-glu-gly at 37°C for 10 minutes. Then 250 µL of coloring agent (contained 15 g trichloroacetic acid and 5 g iron (III) chloride hexahydrate in 100 mL of ddH_2O) was added and centrifuged at 10,000 rpm for 5 min. The supernatant was collected for the measurement of the TG activity.

Finally, lysozyme activity was measured at 450 nm using a suspension of *Micrococcus lysodeikticus* as a substrate in a reaction mixture (Shugar 1952). Hemolymph (10 µl) was mixed with 200 µl of *M. lysodeikticus* in PBS (0.02% (w/v)) at 25 °C. The OD reading was recorded with a spectrophotometer every 6 minutes after adding *M. lysodeikticus*. Lysozyme from white hen egg (Amresco, USA) was used as control.

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

Total RNA was extracted from MGs and HPs using Trizol® reagent (Invitro, USA) according to the manufacturer's instructions. The RNA samples were suspended in 30µl DECP-treated water and kept at -80ºC until use. Total RNA was qualified and quantified using Biospectrometer (Germany). According to the manufacturer's instructions, first-strand cDNA was synthesized from 2 µg of total RNA using M-MuLV reverse transcriptase (Lucigen). The cDNA was subsequently stored at − 20°C.

Real-time PCR was performed using the ABI StepOnePlus™ Real-time PCR system (Applied Biosystem, USA). The amplification was performed in a total volume of 10 µl, containing 1 µl of cDNA template, 5 µl of SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Bio Inc), 0.2 µl of each primer, 0.2 µl of ROX reference dye (50x), and 3.4 µl of DEPC treated water. The real-time PCR program was 95°C for 1 min, followed by 40 cycles of 95°C for the 15s and 60°C for 1 minute. Dissociation and melting curves of amplification products were performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The 2-ΔΔCT method was chosen as the calculation method (Livak and Schmittgen 2001).

Table 1
RT-qPCR primers of immune-related genes

Target genes	Genebank	Primer	Sequence (5'-3')
LGBP (LPS-β-glucan Binding Protein)	GQ228481	F	AGGAACCGGCGGTTTCTT
		R	GGTGTTGGACCAAGGCTTGT
PE (Peroxinectin)	JN572543.1	F	CACTGCTGCCTTCCGTTTC
		R	AGGGCTTGTGGATTATTCTG
proPO (Prophenoloxidase)	AY947400	F	ACACTGAAGGACATAAGGCGAGAT
		R	AGTAGAGTTCCAAGTCGGAGATGCT
Mrß-actin,	AY651918.1	F	CCCGACGGTCACTTGTTC
		R	CGTGGATGCCGCAGGATT

2.5. Statistical Analysis

ANOVA (One-way analysis of variance) was applied to evaluate data obtained from the experiments. Multiple comparisons (Duncan test) were conducted to investigate significant differences among treatments using SPSS (version 17, USA). Results were displayed as the mean ± SD, $p < 0.05$, as the significance level for every test.

3. Results

3.1. Effect of *S. radix* extract on the hemocyte immune responses

The THC data from four treatments recorded for 24 hours after the RGF method can be seen in Fig. 1. Overall, for 24 hours, *S. radix* extract (T1 and T2) raised THC significantly in prawns ($P < 0.05$), while the addition of 10 μg $μl^{-1}$ was the worse treatment for promoting THC *M. rosenbergii*. Moreover, TG activities were significantly increased at T1, T2, and T3 for 24 hours post-RGF, except for a return of T3 to normal level at 24 hours (Fig. 2). The highest activities of TG were observed at 3-hour post-RGF in each treatment but then were decreased over time. Meanwhile, RBs activity of T2 was significantly increased at 3-hour, but no significant differences were found among T0, T1, and T3 until 6-hour post-RGF. However, RBs activity returned to normal at 24-hour post-RGF (Fig. 3). Finally, the T3 had the highest level of lysozyme activity at the first observation, 3 hours after RGF, while the remaining treatments did not show significantly different from the control group ($P > 0.05$). Furthermore, lysozyme activities of all treatments increased 24-hour post-RGF compared with the control group ($P < 0.05$) and reached the peak presented in Fig. 4.

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

3.2.1. hepatopancreas

The expressions of LGBP in *M. rosenbergii* HP were decreased post-RGF in all treatments for 24 hours, except for an increase of LGBP (2.1-fold) at 12 hours post-RGF in T3 (Fig. 5). The down-regulation of PE in T2 was observed as early as 3 hours (0.2-fold) post-RGF (Fig. 6). An increase in PE was only shown 12 hours post-RGF in T3 (non-significant).

3.2.2. midgut

The expressions of LGBP in *M. rosenbergii* MG were down-regulated as early as 3 hours post-RGF at T2 and T3, while T1 expression was not significantly different (Fig. 7). The up-regulation of LGBP was observed in all treatments at 6 hours. Furthermore, an increase of LGBP in MG was also seen at 12 and 24 hours post-RGF in T2 and T1 (2.6- and 5.35-fold, respectively). The expression of proPO was upregulated in all treatments for 3 hours and only T2 for 12 hours (1.77-fold), while at 6 and 24 hours showed no significance among treatments (Fig. 8). The up-regulation of PE in T1 was observed as early as 3 hours (10.64-fold) post-RGF (Fig. 9). An increase in PE also was shown at 12 hours post-RGF in T3 (Fig. 10).

4. Discussion

The majority of immune responses occur in hemolymph, which contains three distinct types of hemocytes: semigranular, granular, and hyaline (Interaminense et al 2019). The granules of the hemocytes synthesize and store numerous immunological compounds until they are delivered into the hemolymph following bacteria or fungi activation molecules, such as peptidoglycan, ß-glucans, and lipopolysaccharides (Sritunyalucksana and Söderhäll 2000).

HP, an endocrine and digestive organ, is responsible for food intake, digestive enzymes secretion, transport, and storage of lipids, glycogen, and minerals, equal to the liver and pancreas organ in vertebrates (Felgenhauer 1992). Moreover, HP, or midgut gland, is also believed to be an important site for expressing immune-related genes in shrimp (Pan et al 2005; Silveira et al 2018). Another critical part is the midgut, vital for pathogen entering, colonization, and transmission. The midgut is formed by a glandular (columnar) epithelium destitute of cuticle and lined with a peritrophic membrane (Dall 1967). During infection, the midgut performs as an area for the expression of immune-related genes (Hauton 2012).

4.1. Effect of *S. radix* extract on the immune responses

4.1.1. Total hemocyte count

THC can vary depending on environmental stresses (Perazzolo et al 2002), infections (Feng et al 2008), immunostimulants (Y. Chen et al 2014), and endocrine activities (Estrada et al 2016). Hemocytes conduct various physiological and pathological functions, such as antigen recognition, phagocytosis, encapsulation, nodule formation, and releasing of humoral factors (Chang et al 2015; Hose et al 1987). In the present study, we evaluated the effects of *S. radix* extract on the THC of *M. rosenbergii* via RGF. One and 5 μg/μl showed the highest THC compared with the control group during 24 h. The similar results described by Wu et al. (Wu et al 2015) showed that the water extract of *Gynura bicolor* could enhance THC of *Litopennaeus vannamei* at 7th -day post-feeding treatment. Noni leaves were also shown to increase *M. rosenbergii* THC at 3rd -day post-feeding treatment (Halim et al 2017). It is supposed that *S. radix* extract can promote hemocyte proliferation in prawns via RGF.

4.1.2. Transglutaminase

Coagulation is initiated by releasing factors and enzymes from hemocytes, which effectively polymerizes the hemolymph clotting protein (Martin et al 1991). Liu et al (2011) reported that, in crustaceans, clotting is mediated through coagulates presented in the plasma and TGs within circulating hemocytes. TG prevents hemocyte loss during the microbial invasion and inhibits their growth in the hemocoel (Fagutao et al 2012). According to Theopold et al. (Theopold et al 2004), hemolymph clotting is responsible for binding and killing pathogens in horseshoe crabs. This process involves inflammatory responses, wound closure, and healing (Theopold et al 2004). In the present study, RGF with *S. radix* extract enhanced TG directly for 24 hours.

Moreover, the concentration of 5μg/μl induced the highest TGs activity. Another research using *Eichhornia crassipes* supplemented feed to enhance the immune system of *M. rosenbergii* also showed a similar result (Chang et al 2013). Indeed, TG activities of prawns fed with *E. crassipes* containing diets were significantly higher than prawns fed with the control diet. Together with an increase in THC, our data support a functional involvement of TG in the acceleration of hemolymph coagulation of *M. rosenbergii*.

4.1.3. Respiratory burst

Increased RBs activity is related to increased bactericidal activity by phagocytes or hemocytes within the cellular immune system (Song and Hsieh 1994). Bactericidal activity is performed by superoxide anion and other reactive oxygen species (ROS) stimulated by RBs activity (Y.-Y. Chen et al 2014). In this study, 5 and 10μg/μl of *S. radix* extract raised RBs activity promptly on *M. rosenbergii* post-RGF. A similar study by Chen et al. (Y. Chen et al 2014) revealed that RBs activity of *L. vannamei* was increased at 24 hours post-feeding with *P. binghamiae* hot-water extract at the concentration of 10μg/g. Furthermore, crude extract of *Cardiospermum halicacubum* leaves supplemented with commercial shrimp pellet enhanced *Penaeus monodon* RBs activity from 48 to 120 hours after feeding (Rajasekar et al 2011). These results suggest that administration of *S. radix* extract by RGF can induce RBs activity of hemocytes in prawns more rapidly.

4.1.4. lysozyme

Lysozyme is found in hemocytes and most shrimp tissues (Burge et al 2007; Mai and Hu 2009; Qiao et al 2013). It is an antimicrobial agent against pathogen invasion (Kaizu et al 2011; Ragland and Criss 2017). It has been determined that an increase in lysozyme activity protects the host by serving as an essential defensive enzyme against infectious pathogens (Ragland and Criss 2017; Saurabh and Sahoo 2008). It catalyzes the hydrolysis of bacterial cell walls following an order from phagocytic cells (De-La-Re-Vega et al 2004; Haug et al 2002; Rojtinnakorn et al 2002). In this study, the RGF of *S. radix* extract enhanced *M. rosenbergii* lysozyme activity in hemocytes as early as 3 hours post-RGF at the concentration of 10 μg/μl. However, the rise of lysozyme at the concentrations of 1 and 5μg/μl were only seen after 12 and 6 hours, respectively. Consistent with our result, increases in lysozyme activity were also seen in the extractions of guava leaves and zingerone in *Penaeus monodon* and *L. vannamei*, respectively (Chang et al 2012; Yin et al 2014). Moreover, lysozyme production was mainly based on hemocytes present in hemolymph (Burge et al 2007; Mai and Hu 2009). These results suggest that the enhanced lysozyme activity of prawns in our study could result from THC increment after RGF of *S. radix* extract. However, the application of *S. radix* could not enhance lysozyme activity in tilapia, *Oreochromis niloticus* (Yin et al 2006). Therefore, further research is necessary to clarify the effects of *S. radix* on different animals.

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

4.2.1 Lipopolysaccharide-and β-1, 3-Glucan-binding Protein

LGBP was mainly expressed in the HP (Pan et al 2005; Yeh et al 2009). LGBP is involved in cellular and humoral defenses using PAMP (pathogen-associated molecular patterns) and stimulates a cascade of responses, such as phagocytosis, clotting activation, antibacterial peptides production, and proPO cascades (Iwanaga 1994; Lai et al 2011; Roux et al 2002; Yeh et al 2009). In our study, the up-regulation of the LGBP gene in HP was only observed at 12 hours post-RGF at the concentration of 10μg/μl. It is reported that LGBP expression was increased in the HP of *L. vannamei* at 72 hours and 7 days post-feeding treatment of diets containing β-1,3-glucan (Wang et al 2008). These results suggest that the difference in LGBP expression was modulated by delivery method, species, and types of immunostimulants. For MG, all treatments showed the up-regulation of LGBP at 6 hours, while at 12 and 24 hours, LGBP was upregulated by 5 and 1μg/μl, respectively. Increases in the expression of LGBP in MG are believed to originate from infiltration of hemocytes but not gut tissue (Silveira et al 2018). Hemocytes perform a migratory behavior to shrimp digestive tissues and contribute to gut immunity (Arts 2006; Silveira et al 2018; Yeh et al 2009). Based on the statements above, we speculated that the increased LGBP in MG was due to their content of hemocytes. Yeh et al. (2009) also indicated the upregulated LGBP expression in HP of *M. rosenbergii* 12 hours after the prawn had received LPS or Poly: IC, but was downregulated in hemocytes (Yeh et al 2009), suggesting that the regulation of LGBP transcription was not tissue-specific.

4.2.2 Prophenoloxidase

The proPO activating system is believed to be an essential innate defense in invertebrate immunity (Charoensapsri et al 2011). Activation of proPO is shown to generate cytotoxic products, melanin

production (melanization), and encapsulation of pathogen (Cerenius et al 2008). The shrimp's proPO expression was found in the hemolymph, gill, stomach, HP, and intestine (Ai et al 2008; Charoensapsri et al 2009; Pang et al 2014). The proPO in various shrimp tissues showed that proPO is used for several functions, including growth encouragement or larval development (Charoensapsri et al 2011), playing a central role in killing and eliminating invading pathogens (Tassanakajon et al 2018). According to Soonthornchai et al (2010), The up-regulation of proPO expression in diverse tissues, including HP and MG, was applied as a hemocyte marker to know hemocyte infiltration into various tissues.

The up-regulation of proPO in MG was observed at 3 hours post-RGF in all concentrations. The proPO expression was decreased in other time points; only 10 μg $μl^{-1}$ still showed significance at 12 hours compared with other groups. The present study believed the up-regulation of proPO in MG was stimulated by infiltrating hemocytes, particularly granular hemocytes (Silveira et al 2018). The granulocytes store and release the proPO system (Johansson et al 2000; Silveira et al 2018). In our study, *S. radix* may stimulate granulocytes circulated to digestive organs (Tanekhy and Fall 2015). This result is consistent with some studies on *Morinda citrifolia* (Halim et al 2017), *Musa acuminate* (Rattanavichai and Cheng 2015), and *Solanum nigrum* (Harikrishnan et al 2011).

4.2.3 Peroxinectin

Peroxinectin is a cell adhesion protein essential to invertebrates for many physiological processes, including immune responses (Johansson 1999). Active PE can bind to integrin receptors on the cell surface to encourage the degranulation process in a positive reaction loop and play a multifunctional agent during pathogen invasion (Sricharoen et al 2005). In the present study, the expression of PE transcripts showed no significant difference in *M. rosenbergii* HP among groups post-RGF. This response of PE was correlated with previous studies that PE expression was not detected in naïve prawn HP (Hsu et al 2006) or during 24 h post bacterial infection (Du et al 2013). Moreover, a recent study has reported non-significant transcription of PE detected in shrimp HP concerning control during 48 hours after *Vibrio campbellii* infection (Burge et al 2009). These data suggest that prawn HP may lack the ability to respond acutely to stimuli. Apart from HP, the expression of PE in MG was increased as early as 3 hours post-RGF in the concentration of 1μg/μl, but was down-regulated in the concentrations of 5 and 10μg/μl and showed a decay pattern after that. According to Silveira et al., (2018) (Silveira et al 2018), hemocytes are present in MG and contribute to the expression of immune-related genes. Moreover, the early induction of shrimp PE upon starvation has been previously reported (Lin et al 2012), suggesting that the early up-regulation of PE in MG can be associated with the gut-associated hemocytes and response of prawns to 1 day-starvation. The decrease of PE was also detected in prawn hemocytes after injection with foreign materials (Hsu et al 2006), implying the most likely explanation for the down-regulation is a decline in the number of PE-expressing cells. On the other hand, the expressions of PE mRNA are uniformly low in the high concentrations of *S. radix* extract, indicating a dose-dependent pattern of prawn in response to the stimulus.

5. Conclusion

The results demonstrated that *S. radix* extract improved the innate immunity of *M. rosenbergii* by increasing the THC, TG activity, RBs activity, and lysozyme activity. Moreover, the extract regulated the expression of immune-related genes that play crucial roles in prawn immunity. In summary, RGF can elicit a robust immune response and appeared as an extremely fast delivery method to investigate the immunostimulant effect of *S. radix* in prawns.

Declarations

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Data availability All the required data are available in the manuscript.

Code availability Not applicable.

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Consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

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Figures

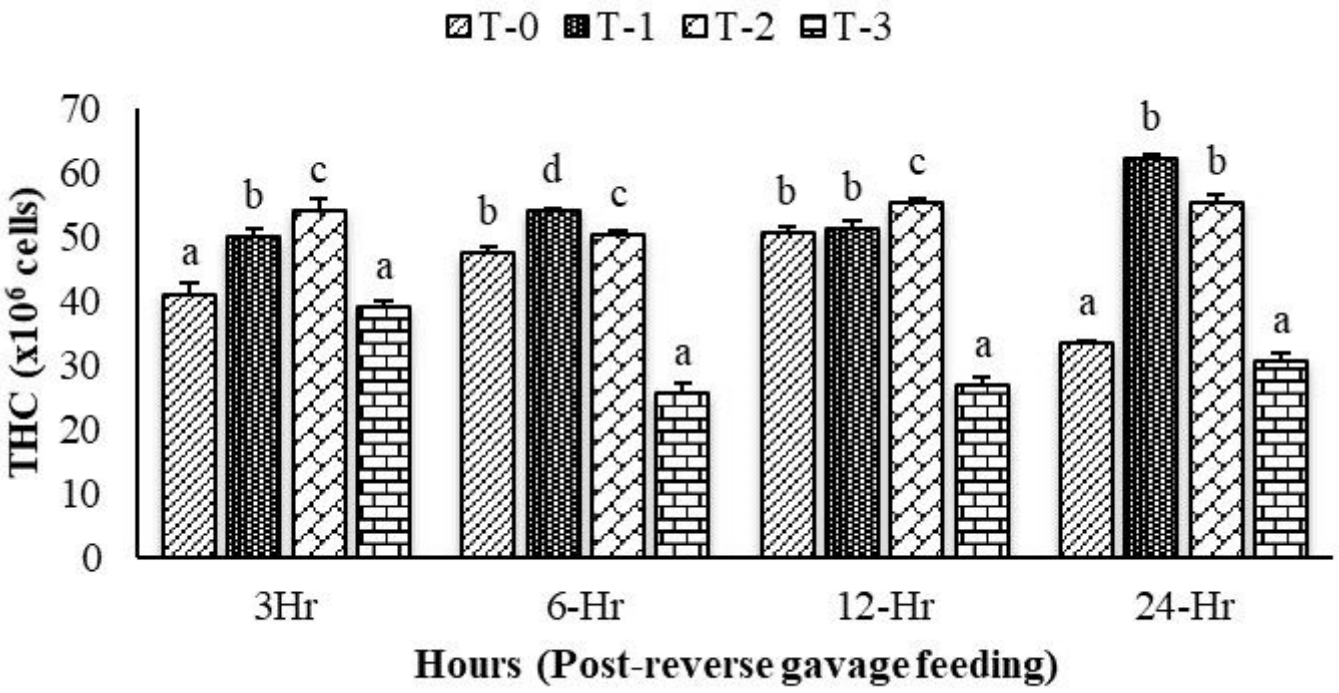

Figure 1

Total hemocyte count 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 treatments.

Figure 2

Transglutaminase activity of *M. rosenbergii* post-RGF feeding with *S. radix* water extract for 24 hours. Data (mean ± SD) with different letters are significantly different (*P*<0.05) among treatment.

Figure 3

Respiratory bursts 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 treatments.

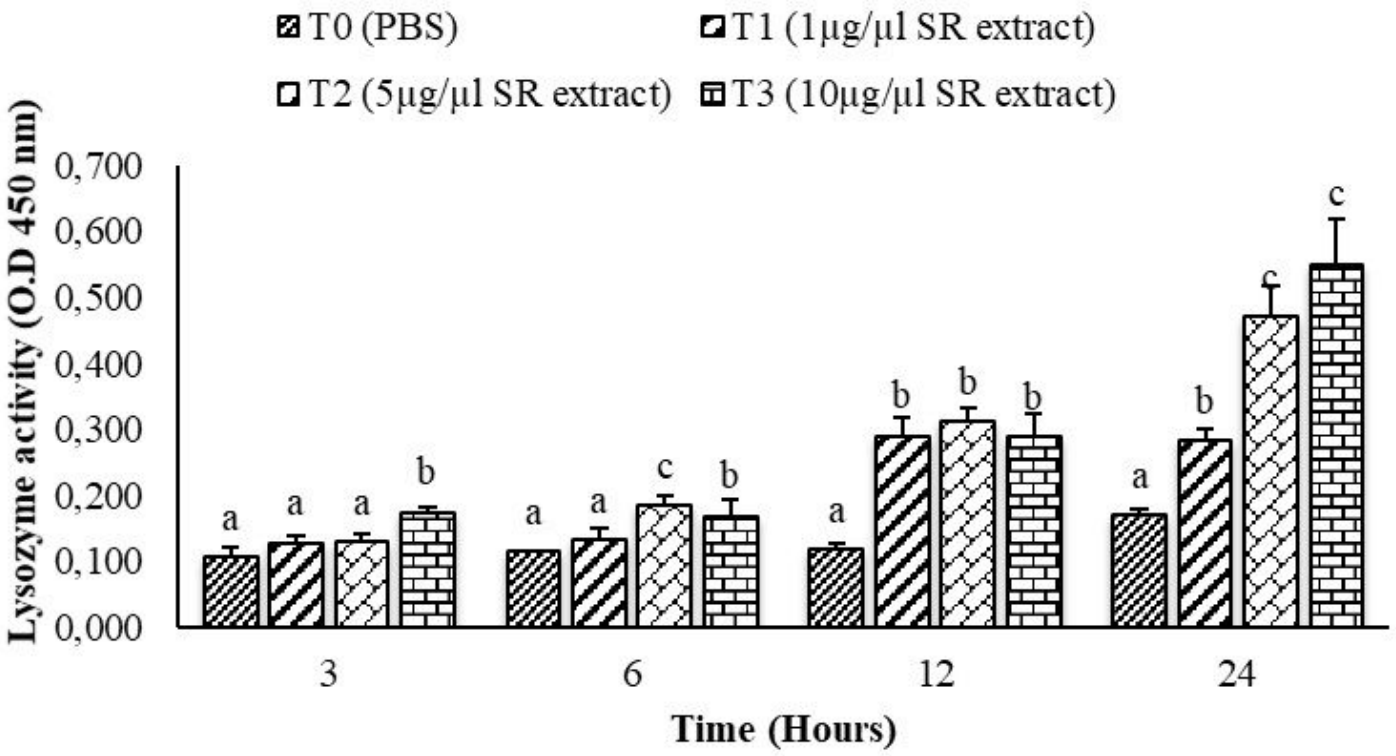

Figure 4

Lysozyme activities of *M. rosenbergii* post-reverse gavage feeding with *S. radix* water extract for 24 hours. Data (mean ± SD) with different letters are significantly different (*p*<0.05) among treatment.

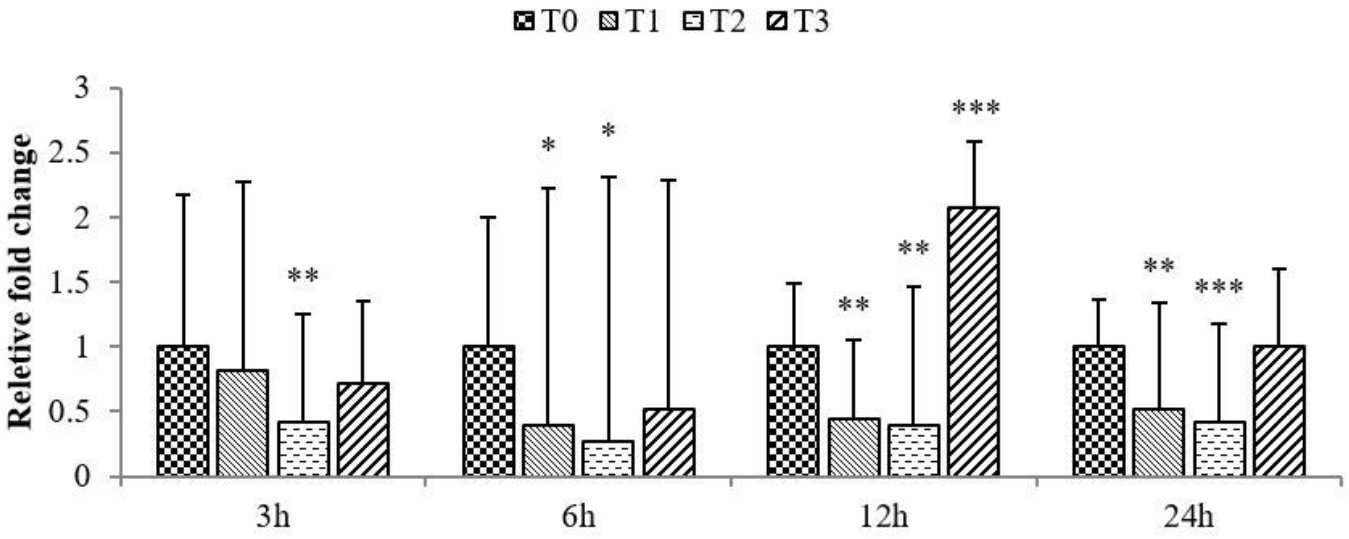

Figure 5

Expression of LGBP in *M. rosenbergii* hepatopancreas post-RGF

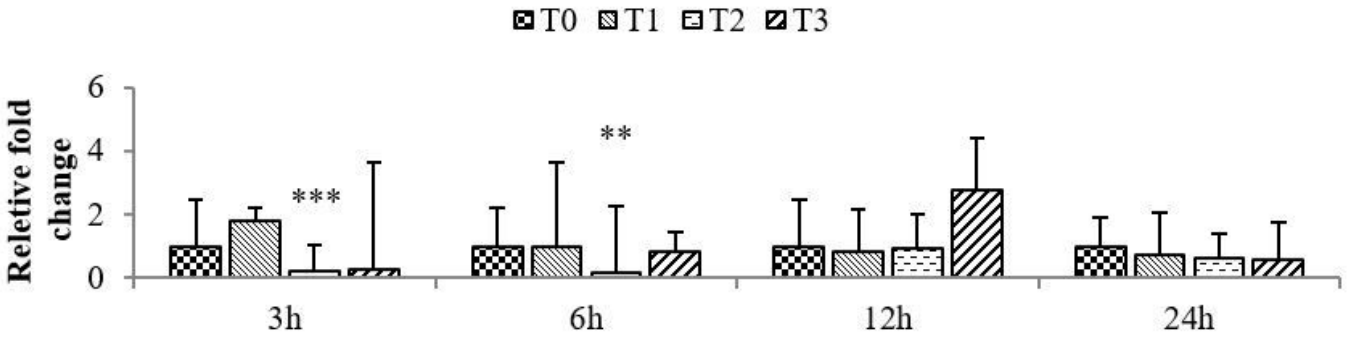

Figure 6

Expression of PE in *M. rosenbergii* hepatopancreas post-RGF

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Figure 7

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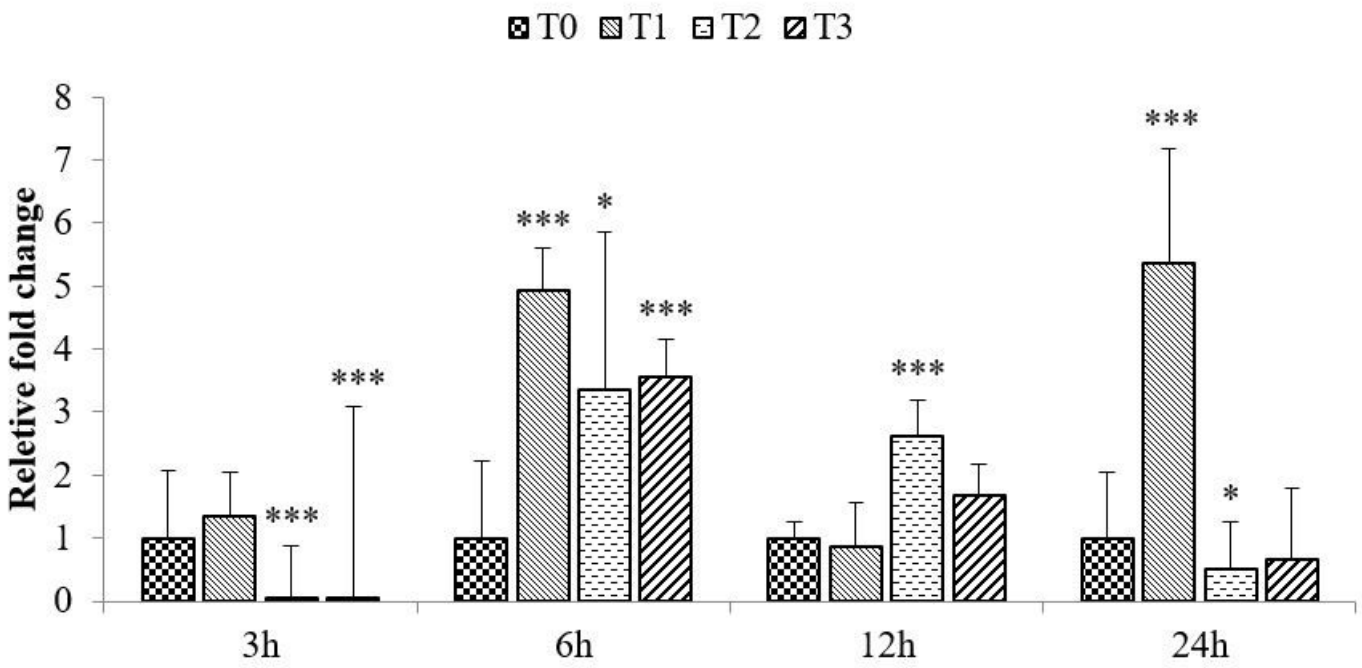

Figure 8

Expression of LGBP in *M. rosenbergii* midgut post-RGF

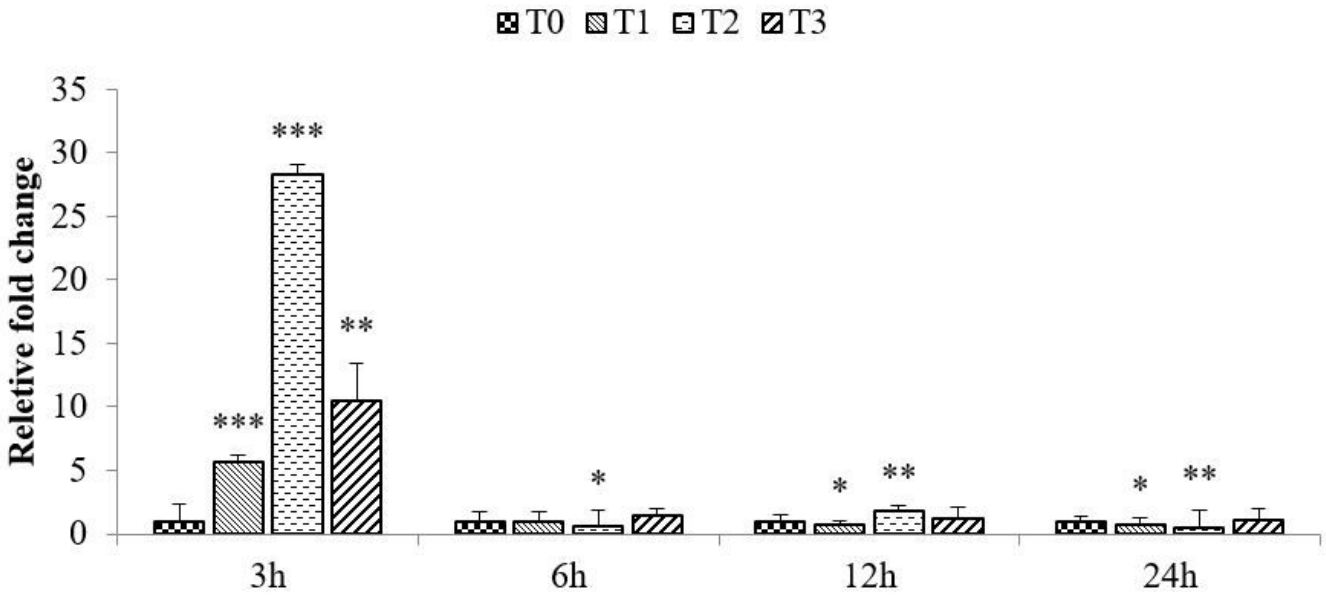

Figure 9

Expression of proPO in *M. rosenbergii* midgut post-RGF

Figure 10

Expression of PE in *M. rosenbergii* midgut post-RGF