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Original Article

Effects of safflower (*Carthamus tinctorius*) extract on serum antibacterial activity of rainbow trout (*Oncorhynchus mykiss*) against *Aeromonas hydrophila*, *Streptococcus iniae* and *Yersinia ruckeri*

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Abstract: In the present study, the effects of safflower (*Carthamus tinctorius)* extract on serum antibacterial activity of rainbow trout (*Oncorhynchus mykiss*) against *Aeromonas hydrophila*, *Streptococcus iniae* and *Yersinia ruckeri* was studied. In this regard, 450 fish with average weight of 100±10 g were stocked into fifteen fiberglass tanks. This experiment consisted of 3 treatment groups (received 50, 100 and 200 mg/kg BW safflower extract via IP injection), one positive control group (just received normal saline) and one negative control group (with no injection). Blood samples were taken at the 3th, 7th and 10th days after the injections, and antibacterial activity of serum were determined *in vitro* using CFU method. The results showed that safflower extract injection had no significant effects on serum anti-bacterial activity against *A. hydrophyla* and *Y. rukeri* during 10 days post injection. However, in the fish receiving 100 mg/kg safflower extract, serum bactericidal activity against *S. iniae* was significantly higher than the other groups. This study demonstrated that safflower extract at the doses of 50-200 mg/kg via IP injection did not cause significant changes in serum *antibacterial activity against A. hydrophila* and *Y. ruckeri*, but injection of 100 mg/kg extract led to an increase in the serum antibacterial activity against *S. iniae*, in rainbow trout.

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Introduction

Nowadays rainbow trout is one of the most important species in cold-water aquaculture of the world. An increasing trend towards the breeding and reproduction of this fish has raised the incidences of various diseases, including the bacterial ones. Bacterial pathogens are the most common pathogens in aquaculture, causing many problems and damages. Improving the level of immunity along with the use of immunostimulants is a useful and cost-effective method to increase fish resistance to pathogens and to prevent the formation of drug resistance bacteria.

Every year, bacterial diseases are responsible for the loss in many trout farms. Treatment with antibiotics is one of the common methods to control these diseases, while vaccinations would be also useful in some cases. Due to the pathogens' resistance to antibiotics, some limitations have been established against their application; besides, antibiotics are also responsible for killing the beneficial bacteria in the host's digestive tract (Aoki, 1990). Prophylactic methods are superior to therapeutic methods from the economic aspects, thus methods for improving fish immunity and resistance to diseases are more suitable than antibiotic application. Due to the presence of effective natural compounds in medicinal plants, the use of medicinal plants and their natural derivatives has increased recently. Most of these compounds have no side effects on fish and consumers, resulting in remarkable advantages compared to chemical drugs (Velag and Studlla, 2005).

On the other hand, some fish bacterial diseases are important in human health and hygiene. An example is the infection with *A. hydrophila*, found in association with fish and crustacean. Human infections with *Aeromonas* spp. are often observed in

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people with a weak immune system, and localized wounds, swelling and gastroenteritis are some of the most common signs of the infection (Moyer, 1987; Lowry and Smith, 2007). *Streptococcus iniae* is another zoonotic bacterium that can cause serious problems in humans, with the most clinical signs in the patients being endocarditis, cellulitis, meningitis and systemic arthritis (Weinstein, 1997).

To achieve sustainable development in aquaculture industry, it is necessary to find methods to reduce high costs of disease treatments in ponds. In this regard, it is essential to follow quarantined principles and prevent the spread of the pathogens. In addition, improving the fish resistance against pathogens and environmental stress is effective method with low cost. For this purpose, use of oral or injectable herbal extracts with antioxidant or antimicrobial properties would be beneficial; besides, some plants increase growth performance, immunological parameters and environmental stress resistance in fish (Velag and Studlla, 2005; Sahu et al., 2008).

Safflower (*Carthamus tinctorius*), a member of the Compositae family (Villa, 2017), has been of particular interest to humans for the past 2500 years for its therapeutic effects (Li, 2013). Nowadays more than 200 compounds have been extracted from this plant. Many therapeutic effects on humans and laboratory animals, such as anti-hypoxia, liver anti-fibrosis, antioxidant, stimulate the immune system and anti-tumor and anti-inflammatory effects have been reported for this extract (Zhou, 2014; Fan, 2009). However, in this regard, there are some researches about the effects of this extract on fish (Dadras et al., 2016; Choi et al., 2010). In the present study, antibacterial effects of safflower extract injection have been studied against some fish pathogenic bacteria (*A. hydrophila*, *Y. ruckeri* and *S. iniae*) in rainbow trout.

Materials and Methods

A total numbers of 450 rainbow trout with an average weight of 100±10 g were transferred to the Fisheries Research Station of Gharehsou (Bandar-Turkmen, Golestan Province, Iran). The fish were distributed into fifteen 500-L fiberglass tanks containing 200 L water at a flow rate of 2 L/min. Acclimation was carried out for one week, during which the fish were fed with a commercial diet (3% of mass weight daily). This experiment was consisted of 5 treatments: negative and positive controls, 50, 100 and 200 mg/kg extract groups. To prepare the injectable extract, ethanolic extract was made from dried petals of safflower. Briefly, the petals were washed (with cold sterile water), dried and grounded. Five liters of 96% ethanol were added to 500 g of the dried powder as a solvent in a ratio of 1:10. Later, the mixture was transferred to a dark container and was mixed well. After that, the container was tightly closed and shaken for seven days at room temperature to ensure well-mixing. After seven days, the supernatant was passed through a filter paper to remove any insoluble suspended particles; after which the filtered supernatant was dried using an oven and finally the residual material was packed in sterile containers as ethanolic extract (Harikrishnan, 2009). This extract was dissolved in 0.9% sodium chloride at 40°C to prepare the injectable solution for intraperitoneal injection at the dosages of 50, 100 and 200 mg/kg of fish weight. Each dosage was injected in a 0.1 ml solution to the fish of three tanks. Positive control was injected by the 0.9% sodium chloride solution. A negative control group, consistent of three tanks, was assigned with no injection. For injecting, the fish were netted and anesthetized in 100 ppm eugenol. The extract was injected intraperitoneally using an insulin syringe. The injected fish were also transferred to separate 500-L tanks filled with 200-L of water with constant flow rate.

Blood samples were taken three, seven and ten days after injection. Six fish were sampled for treatment at each sampling time. For blood sampling, the fish were netted and anesthetized with 100 ppm eugenol. The blood samples were collected from the caudal vein using 2-ml syringes. Sera was obtained by centrifugation at 6000 rpm for 7 min and stored at -80°C for analysis.

Three pathogenic bacteria including *A. hydrophila*, *Y. ruckeri* and *S. iniae* were used to assess the serum

bactericidal activity. The bacteria were first cultured on nutrient agar medium. After 48 hrs of incubation at 25°C, the bacteria were collected from the second passage culture according to Sahu (2008) and suspended in 0.9% sodium chloride solution with one Optical Density (OD) at 640 nm. Each of the bacterial suspensions was diluted three times and used as a main sample of bacteria. 100 µL of serum was mixed with 100 µL of the bacterial suspensions and then incubated at 37°C for 4 hrs after that 6-fold serial dilutions were prepared from the mixture (according to the National Iranian Standard, No. 3-8923, 2006).

100 µL of all dilutions for each treatment were inoculated in nutrient agar plates and after 48 hrs resting at room temperature, the viable total count calculated for each treatment based on Colony Forming Unit (*CFU*). For estimating the total count of bacterial suspension, 100 µL of bacteria was added with 100 µL sterilized 9% sodium chloride solution and after 4 hrs at 37°C, a viable total count of each bacterium was determined.

Statistical analyses were implemented using the SPSS version 22.0 for windows (IBM SPSS Inc., Chicago, IL, USA). The normality of the data and homogeneity of variances were confirmed by Shapiro-Wilk and Levene tests. Data of the serum bactericidal activities were analyzed by one-way analyses of variance (ANOVA) followed by Duncan's test. A statistical significance was accepted at $P<0.05$ levels.

Results

Data of bactericidal activity against *A. hydrophyla* are presented in the Figures 1-3. The bacterial load has significantly decreased in serum treatments compared to that of the bacterial control (Figs. 1-3). However, there was no significant difference among negative and positive controls and the extract treated sera at any sampling times.

Data of the bactericidal activity against *Y. rukeri* are presented in Figures 4-6. Negative and positive controls had significantly lower bactericidal activity compared to the bacteria control at the third day (Fig. 4). However, the extract-treated fish bactericidal activities were similar to the bacteria control but not

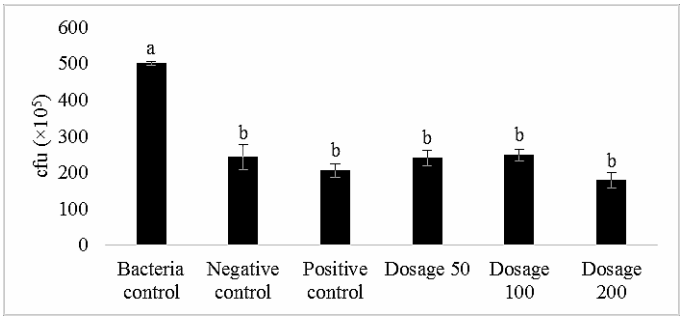

Figure 1. Serum bactericidal levels of treatments compared to control plate containing *Aeromonas hydrophila* on the third day. Different letters above the bars show significant difference.

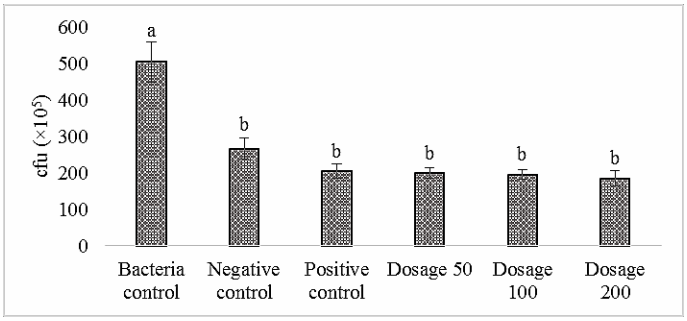

Figure 2. Serum bactericidal levels of treatments compared to control plate containing *Aeromonas hydrophila* on the seventh day. Different letters above the bars show significant difference.

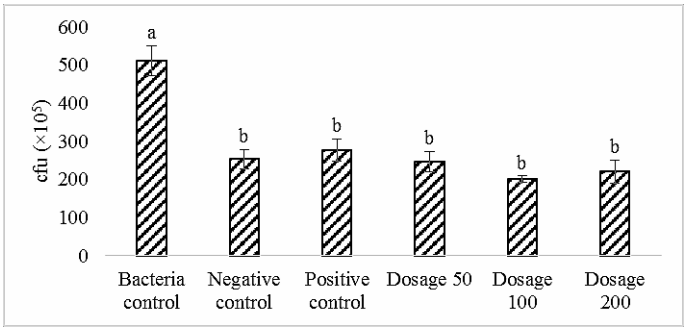

Figure 3. Serum bactericidal levels of treatments compared to control plate containing *Aeromonas hydrophila* on the tenth day. Different letters above the bars show significant difference.

significantly different compared to negative and positive controls (Fig. 4). After, seven days, the serum treatments excluding the 50 and 200 dosages had a lower bacterial load compared to the bacteria control, however, there was no significant difference among the serum treatments (Fig. 5). At the tenth day, the bacterial load significantly decreased in serum treatments compared to that of the bacterial control (Fig. 6). However, there was no significant difference among negative and positive controls and the extract treated sera.

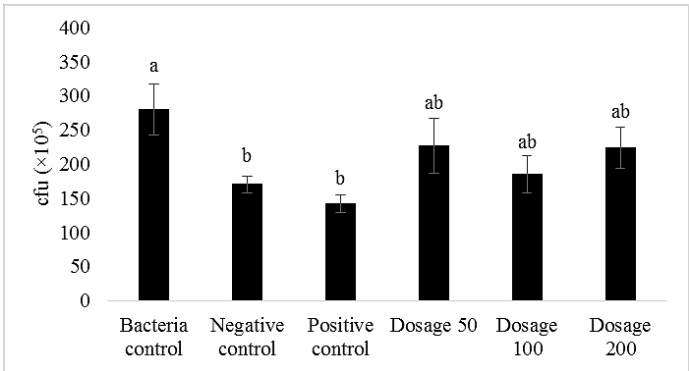

Figure 4. Serum bactericidal levels of treatments compared to control plate containing *Yersinia ruckeri* on the third day. Different letters above the bars show significant difference.

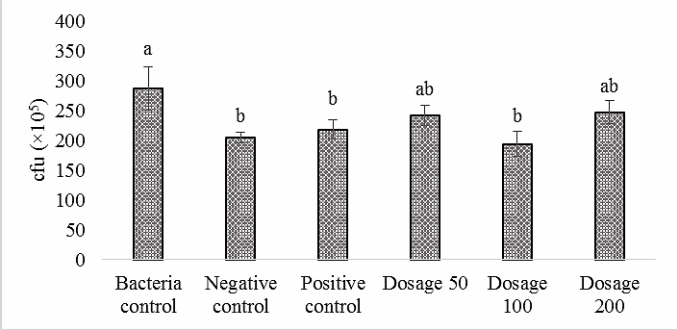

Figure 5. Serum bactericidal levels of treatments compared to control plate containing *Yersinia ruckeri* on the seventh day. Different letters above the bars show significant difference.

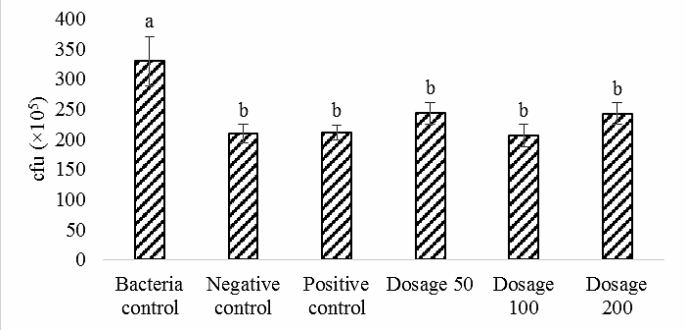

Figure 6. Serum bactericidal levels of treatments compared to control plate containing *Yersinia ruckeri* on the tenth day. Different letters above the bars show significant difference.

Data of bactericidal activity against *S. iniae* are presented in the Figures 7-9. At the third day, the bacterial load of the serum treatments was significantly lower than the bacteria control; however, there was no significant difference among the serum treatments (Fig. 7). After, seven days, the serum treatments excluding the dosage of 200 had lower bacterial loads compared to the bacteria control while the bacterial load of the dosage 100 was significantly lower than the other serum treatments. (Fig. 8). At the

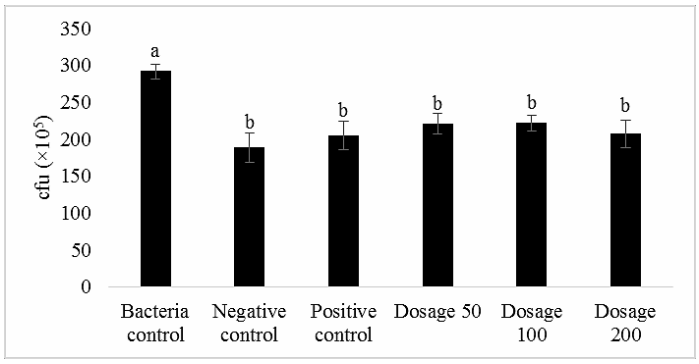

Figure 7. Serum bactericidal levels of treatments compared to control plate containing *Streptococcus iniae* on the third day. Different letters above the bars show significant difference.

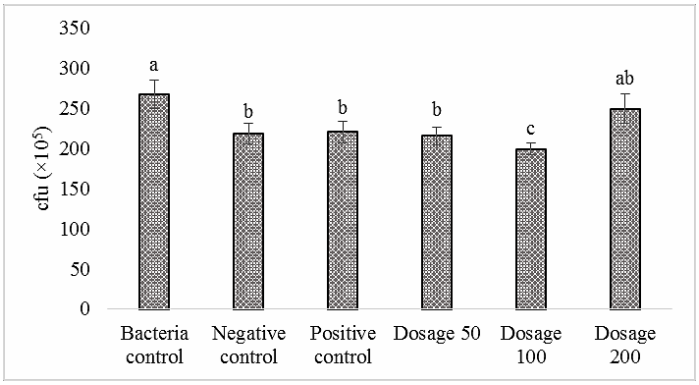

Figure 8. Serum bactericidal levels of treatments compared to control plate containing *Streptococcus iniae* on the seventh day. Different letters above the bars show significant difference.

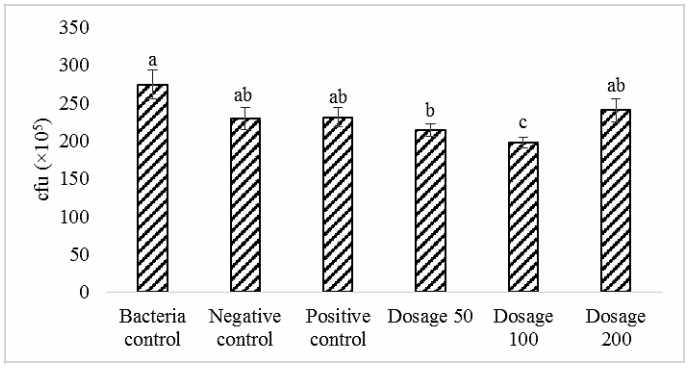

Figure 9. Serum bactericidal levels of treatments compared to control plate containing *Streptococcus iniae* on the tenth day. Different letters above the bars show significant difference.

tenth day, the bacterial loads of dosages 50 and 100 were significantly lower than the bacteria control, while the other treatments had bacterial loads similar to that of bacteria control (Fig. 9).

Discussion

Under stressful conditions, microbial pathogens, especially bacteria can make problems in fish farms (Zorrilla, 2003). This situation leads to a reduction in

the fish growth as well as economic losses. On the other hand, some fish diseases can also be transmitted to humans in the form of zoonotic diseases. Therefore, the control of fish pathogens in farms is necessary and cost effective. In the present study, safflower extract effects on serum antibacterial activities against three fish pathogens, *A. hydrophila*, *S. iniae* (zoonotic bacteria) and *Y. ruckeri* have been investigated.

Streptococcus iniae, is one of the important zoonotic bacteria because of its serious problems and clinical effects on humans. Aged persons are more susceptible to the infection, and are infected via an external wound or ulcer; cooking can lead to the elimination of the bacterium (Agnew, 2007; Sun, 2007; Weinstein, 1997). In addition, *S. iniae* the cause of streptococcusis, is one of the main diseases in aquatic industry in cold/warm and fresh/salt water species. Consequently, due to economic losses in recent years, this disease has been recognized as a one of the most dangerous bacterial diseases among the fishes, resulting to significant losses in many cultivating species (Dadson, 1999; Agnew, 2007; Austin, 2007).

Red mouth disease (RMD) or yersiniosis, is another common bacterial disease that is caused by *Y. ruckeri* resulting to high annual losses in cold-water fish farms throughout the world (Davies, 1989; Zorriehzahra, 2012). This bacterium may cause a large mortality in a wide variety of fish species, also it may cause health problems in humans (Xia, 2004; Poobalane, 2010; Zhou, 2010). Yersiniosis has been also reported in rainbow trout farms of Iran for the first time in 1999, where six bacterial strains were isolated from the infected fish (Soltani, 2014). In many cases, drug therapy for this disease was not successful due to antibiotic resistance (Khushiramani, 2007); however, no human infections have been reported for this bacterium.

Aeromonas hydrophila is a gram-negative bacterium, which may cause problems in humans especially in the patients with a weak immune system. The patients were diagnosed with external swelling wounds and gastrointestinal problems (Moyer, 1987; Lowry and Smith, 2007), therefore, the transmission of such bacterium to humans should always be taken into consideration. In aquaculture, this bacterium may cause high mortality in a wide variety of fish such as common carp, catfish, tilapia, eel, goldfish etc. (Xia, 2004; Poobalane, 2010; Zhou, 2010). This disease causes different abnormal symptoms in fish such as hemorrhagic septicemia, ascites, ulcers, and exophthalmia (Khushiramani, 2007). After some disease incidences and in some cases, antibiotic therapy is not easy and always successful, therefore, anti-Aeromonas vaccines are administered in some cases (Khushiramani, 2007; Poobalane, 2010; Sahoo, 2011).

In order to maintain hygiene, the reduction of antibiotics consumption and the production of safe foods, the use of certain medical herbs that have numerous antibiotic properties was suggested that also elevate body immune activities. In some cases, it can also be used as a vaccine adjuvant, to reduce cost of vaccinations and increase effectiveness of vaccines by injection (Reverter, 2014).

There are several studies on effects of safflower on laboratory animals such as mice. Hepatoprotective effects of this extract have been previously recorded in laboratory mice (Asgari, 2012; Rahimi 2009). These studies showed that intraperitoneal injection of the safflower extract in mice led to significant reductions of hepatocyte damage in exposure with Alloxan poisoning. Despite the reports about improvement of the immune system by safflower extract, there are some reports showing impaired immune systems as a result of safflower extract administration. For instance, Louei Monfared and Salati (2012) reported that intraperitoneal injections of safflower extract at the doses of 1.4 and 2.8 mg/kg induced toxic changes in the placental structure, weight, and thickness of the placenta in the mice during pregnancy, resulting in a decrease in survival rate of neonates during 42 days after birth. IP administration of 50-450 mg/kg.day of safflower extract for 6-8 days decreased serum lysozyme concentration and phagocytic functions of both peritoneal macrophages and peripheral leukocytes in mice (Al-Snafi, 2015, 2016). In fish, Dadras et al.

(2016) demonstrated that dietary administration of 1-2% safflower resulted in a significant increasing in immune function and led to an increased activity of lysozyme, serum alternative complement (ACH50) activities and the number of white blood cells (WBC) in beluga (*Huso huso*).

Such aforementioned contradictions suggest that the effects of safflower extract on immune components depend on experimental conditions, such as experimental animal and rout of administration. According to the present study, injection of the safflower extract does not lead to increased serum bactericidal activity against *A. hydrophila* and *Y. Ruckeri* in rainbow trout, but it improves serum antibacterial activity against *S. iniae* at the doses of 50 and 100 mg/kg during 7 and 10 days. These results may be related to difference in cell wall structures of bacteria. In fact, *S. iniae* is a gram-positive bacterium while the other two bacteria are gram-negative. Therefore, further studies are needed to address the potential effects of bacteria type and rout of the extract administration on different immune components and bactericidal activity.

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چکیده فارسی

اثرات عصاره گلرنگ (*Carthamus tinctorius*) بر خاصیت ضد باکتریایی سرم در قزل آلای رنگین‌کمان (*Oncorhynchus mykiss*) بر علیه باکتری‌های *Aeromonas hydrophila*، *Streptococcus iniae* و *Yersinia ruckeri*

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چکیده:

در بررسی حاضر اثرات عصاره گلرنگ (*Carthamus tinctorius*) بر خاصیت ضد باکتریایی سرم خون قزل‌آلای رنگین کمان بر علیه باکتری‌های *Aeromonas hydrophila*، *Streptococcus iniae* و *Yersinia ruckeri* مورد مطالعه قرار گرفت. برای این منظور تعداد ۴۵۰ نمونه ماهی با میانگین وزنی ۱۰±۱۰۰ گرم در ۱۵ تانک فایبرگلاس تقسیم شدند. در این راستا سه گروه تیمار (به‌ترتیب به میزان ۵۰، ۱۰۰ و ۲۰۰ میلی‌گرم/کیلوگرم عصاره گلرنگ از طریق تزریق داخل صفاقی دریافت نمودند)، یک گروه کنترل مثبت (تنها با سرم فیزیولوژی از طریق داخل صفاقی مورد تزریق واقع شدند) و یک گروه کنترل منفی (بدون تزریق) در نظر گرفته شد. نمونه‌های خون در روزهای ۳، ۷ و ۱۰ پس از تزریق اخذ گردید و خاصیت ضد باکتریایی سرم آنها برای باکتری‌های یاد شده بر روی محیط کشت بر اساس روش CFU ارزیابی گردید. بر اساس نتایج تزریق عصاره گلرنگ هیچ تاثیری بر خاصیت ضد باکتریایی سرم خون برای باکتری‌های *A. hydrophila* و *Y. ruckeri* نداشت. در ماهیان که با دوز ۱۰۰ میلی‌گرم/ کیلوگرم عصاره گلرنگ مورد تزریق واقع شدند، در روزهای ۷ و ۱۰ پس از تزریق خاصیت ضد باکتریایی سرم برای باکتری *S. iniae* به‌طور معنی‌داری در مقایسه با سایر گروه‌ها افزایش داشت. در نهایت بر اساس بررسی حاضر تزریق داخل صفافی عصاره گلرنگ در غلظت‌های ۵۰ تا ۲۰۰ میلی‌گرم/کیلوگرم منجر به تغییر معنی‌دار در خاصیت ضد باکتریایی سرم خون قزل‌آلای رنگین‌کمان برای علیه باکتری‌های *A. hydrophila* و *Y. ruckeri* نگردید، اما تزریق ۱۰۰ میلی‌گرم/کیلوگرم عصاره گلرنگ منجر به افزایش خاصیت ضد باکتریایی سرم خون قزل‌آلای رنگین‌کمان بر علیه باکتری *S. iniae* گردید.

کلمات کلیدی: خاصیت ضد باکتریایی، گلرنگ، سرم، تزریق.