Effects of Dietary Marjoram, Origanum Majorana on Non-Specific Immune Response and Resistance of Common Carp, Cyprinus Carpio against Staphylococcus Lentus

Asaa H. Kadhim[1], Khalidah S. Al-Niaeem[2*], Abdulkareem T. Yesser[3]

[1]Basrah Agriculture Directory, Basrah, Iraq.

[2*]Department of Fisheries and Marine Resources, College of Agriculture, University of Basrah, Basrah, Iraq. E-mail: kalidah_salim@yahoo.com, ORCID ID: https://orcid.org/0000-0001-7236-6464

[3]Department of Marine Vertebrates, Marine Science Center, University of Basrah, Basrah, Iraq.

Abstract

The current study aimed to evaluate the effect of marjoram *Origanum majorana* extract on non-specific immunity and survival rate in common carp fish infected with *Staphylococcus lentus*. A total of 120 fish, with an average weight of 16.31–16.48 g, were divided into four treatments, each with three replicates and ten fish per tank (30 fish for each treatment). and The first treatment was fed a diet free of marjoram extract. The second treatment was fed on a diet containing 0.5% of marjoram extract, the third treatment was 1%, and the fourth treatment was 1.5%. The fish were fed twice a day at a rate of 3% of their total weight until the end of the experiment for 56 days. 8 fish from all treatments were used as samples to measure the immunological parameters: Nitroblue tetrazolium activity (NBT%), Myeloperoxidases activity (MPO%), phagocytic activity (%) and lysozyme activity (unit/ml). For the *S. lentus* infection challenge, 32 fish from all treatments were used. the The fish in the experimental groups were injected intramuscularly with 0.2 ml of the suspension containing *S.lentus* bacteria at a concentration of 1×107 ml/CFU. After 14 days, the immunological parameters were measured again, and the results after the feeding period showed that the third treatment (1.5%) significantly increased ($p < 0.05$) NBT, MPO, phagocytic activity, lysosomal enzyme activity, and survival rate compared with the control treatment followed by the second treatment (1%), and the first (0.5%). The results showed that the third treatment (1.5%) had a higher resistance to *S.lentus* challenge compared to the other treatments. The results indicate that the addition of aqueous extract of marjoram at a concentration of 1.5% to the diet of cultured carp fish can enhance the protection against any possible infection with *S.lentus* bacteria.

Keywords: Marjoram, Non-specific Immunity, Common Carp, *Staphylococcus*.

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INTRODUCTION

The disease is a major problem causing heavy losses to fish farmers all over the world (1, 2). In order to achieve optimal production, during culture operations, better diagnostic and therapeutic precautions must be taken, and there should be more interest in comprehending fish diseases so that they can be prevented or treated.

(3, 4). Bacterial diseases cause many fatalities in farmed fish, most of which are littermates in natural conditions, but become opportunistic diseases when invade the host tissues (5). The use of antibiotics and chemicals is one of the most common disease control strategies in aquaculture (2, 6). It is often used by fish farmers in large quantities to maintain the health of the fish (7).

However, excessive and indiscriminate use led to the emergence of bacterial strains that are resistant to treatments that are often of a wide spectrum for many species (8).

Eating processed fish may pose a serious threat to human health and cause many health problems such as allergies, poisoning, and others (7). Despite the effectiveness of vaccines in controlling different diseases, they have not had much success (9) due to the difficulty of manufacturing vaccines for multiple strains and the difficulty of applying the vaccine in addition to its high cost (10), therefore, the need for use alternatives to control disease and reduce side effects from antibiotic use and chemotherapy.

The use of plants is the oldest form of health and care known to human. It is effective as anti-stress, growth-promoting, appetite-stimulating, and effective immunostimulant, as well as possessing anti-microbial properties due to the presence of biologically active substances (11). Considered natural immunostimulants for fish that increase innate immunity as biocompatible, biodegradable and safe for the environment and human health (12). In addition, it can be obtained due to its

availability and cheapness, and it can act against a wide range of pathogens (13). Marjoram (also called Sweet majorana) is a perennial aromatic herb belonging to the Lamiaceae family, which includes more than 200 genera and about 3,500 species distributed all over the world.The Mediterranean basin is the original place of it, especially Egypt. Its cultivation has spread in many countries such as India, France, Hungary and the United States for its distinctive flavor and aroma (14). It contains many compounds, including flavonoids, tannins, glucosides, alkaloids and volatile oils (15). It is characterized by its many uses and health benefits for humans, as it was used to improve digestion efficiency, anti-fungal, anti-bacterial, anti-viral, and anti-inflammatory. As well as its use in many common diseases such as food poisoning, typhoid, malaria and influenza (16). Marjoram and Marjoram oil have been used as preservatives for many food products for their role as antimicrobial and antioxidant as well as added to poultry feed to improve growth performance and feeding efficiency of poultry (17, 18, 19, 20, 21, 22, 23). *S. lentus* is one of the animal pathogens that have been isolated from a wide range of pets, farm animals and their products (24, 25). Mastitis caused in goats and sheep (26). In rare cases, the infection spreads to humans and may cause severe infections such as septic shock, endocarditic peritonitis, urinary tract infection, endophthalmitis, and wound infection (27, 28). *S. lentus* has been reported in many types of fish and marine products on the market (29, 30). And isolated by (31) associated with *Aeromonas hydrophilia* from common carp fish in farms north of Basra. And also indicated that both types are considered pathogens affecting aquaculture operations, which leads to a high rate of mortality in cultured ponds. The current study aimed to evaluate the effectiveness of the aqueous extract of marjoram in improving the immune aspect and general health of common carp fish and to test its resistance to experimental infection with *S. lentus*.

Materials and Methods

Preparation of aqueous extract of marjoram

The marjoram plant was obtained from the local markets in Basrah governorate, and the method mentioned by (32) was used to prepare the aqueous extract. Plant leaves have been washed, dried in the sun, then ground with an electric grinder and pass through a sieve with 0.4 mm meshes.

Weight 50 g of the powder were then added to 250 ml of distilled water in a 1 liter glass beaker the mixture is placed in a vibrating incubator at 35 °C for 24 hours. The mixture is filtered twice: once with medical gauze and once with Whatman no. 1 filter paper to remove impurities the extract was kept frozen until use.

Bacteria isolation and activation

Bacteria were isolated from the skin and under the fins of infected fish using the previously mentioned dissemination method (33). Phenotypic tests were carried out to diagnose bacteria using gram staining method (34). The isolated bacteria were diagnosed using VITEK 2 system. To prepare the bacterial culture at 24 hours of age. Swabs were taken from the bacterial culture by loop bacterial vector and placed in test tubes containing liquid nutrient medium N.B after autoclave sterilization and incubated at a temperature of 37˚C for 24 hours. Then it was compared with standard McFarland tubes prepared according to (35). The reading was taken by a spectrophotometer with a wavelength of 600 nm and the bacterial suspension was compared with the MacFarland reading. The turbidity was noted. When the two readings are equal, this indicates that the number of bacteria has become 10^8/ml. The inhibitory activity test (drilling method) was used based on (36).

Fish samples and feeding experiment

120 common carp fish were brought from the earthen ponds of the aquaculture unit of the College of Agriculture, University of Basrah in Al-Hartha. The fish were transferred to the laboratory and sterilized using saturated saline solution and acclimated for 14 days. Fish were weighed and distributed in 12 tanks, 10 fish per tank (30 × 40 × 60 cm). Fish weights ranged between 16.31-16.48 g. Environmental parameters for water quality monitoring were measured with a Taiwanese-born environmental parameters meter (Multimeter), the temperature was (24.5-25.5˚ºC), dissolved oxygen (6.5- 7.0 mg/l), pH (7.45- 7.46), salinity (3.03-3.06 PSU). Fish of all treatments were fed twice daily at a rate of 3% of the average body mass for 56 days. The first treatment was fed on a control diet (0%), The second treatment was fed on a diet supplemented with 0.5% of marjoram extract, the third treatment was 1%, and the fourth treatment was 1.5%. Fish were weighed every two weeks and diet amounts were adjusted based on weight gain.

Infection challenge

After 56 days of feeding, 32 fish were prepared to challenge infection. Distributed to 8 tanks with two replicates for each treatment (4 fish for each tank). The fish was injected in the muscle with 0.2 ml of the bacterial suspension, while the control samples were given a physiological solution. Following infection, the fish were watched for 14 days while being fed diets containing marjoram extract and any external symptoms were noted.

To measure immunohistochemical parameters, blood was collected by drawing blood from the myocardial area immediately after anesthetizing fish using cloves *Syzygium aromaticum*.

Measurement of non-specific immunoassays

Total Myeloperoxidase (MPO) content in serum was measured using (37). Reduction test (NBT) was performed

as to form formazon as a measure of superoxide anion (O^{-2}) production according to the method described by (38). Phagocytic activity was detected using *S.aureus* bacteria using the method described by (39), and to measure lysozyme activity in fish serum, the turbidimeter method described by (39).

Relative Percentage Survival (RPS)

The average mortality data (%) was used to calculate the survival rate after 14 days of the experimental infection, based on (40) as follows:

Survival rate (RPS) = 1 - [(mortality rate (%) in the treatment) ÷ (mortality rate (%) in the control treatment)] ×100%

Statistical analysis

The statistical program SPSS (V. 20) was used to test the differences between the means for all tests using the least significant difference R.L.S.D, at a significance level of 0.05.

Results and Discussion

Inhibitory activity test against S. lentus

Several studies have shown that plant extracts inhibit bacterial growth, but was activity varies. It was described as either strong, moderate or weak (41). Fig. (1) shows the mean diameters of inhibition for *S. lentus* bacteria, as three concentrations of marjoram (0.5, 1, and 1.5%) were used. The results showed that the concentration of 1.5% gave the highest average diameter of inhibition, which was 17.0 mm, while the concentration of 0.5% gave the lowest average diameter of inhibition of bacteria.

One of the important properties of aqueous extracts and its components is ability to resist water, which enables to break down the lipids in the bacterial cell membrane, which leads to the destruction of cell structures and making more permeable. Thus, the exit of molecules and ions will lead to the death of the bacterial cell (42).

Al-Samarrai et al. (43) showed that the aqueous extract of marjoram used against two types of cholera bacteria (Ogawa and Inaba) gave the inhibition zone 13 and 9, respectively, at a concentration of 100 mg/ml, while salmonella bacteria did not show any effect even at a concentration of 200 mg/ml. Al-Turki et al. (44) point out that the aqueous extract of marjoram gave an inhibition area of 13 mm for *E.coli* and 16 mm for *Bacillus subtilis*, while (45) clarified that the aqueous extract of marjoram gave an inhibition zone of 11 mm against *E. coli* and 10 mm against *Bacillus*.

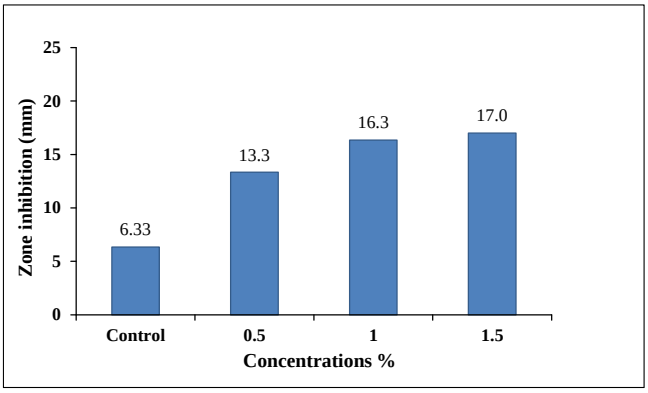

Figure 1: The diameter of the inhibition zone (mm) using three concentrations of aqueous extract of marjoram against S. lentus bacteria

Non-specific immunological parameters

Fish depend on the innate immune response that is Non-specific in preventing infection as it is a critical initial component due to the slow proliferation of lymphocytes and the limited antibodies that delay the adaptive immune response (46). MPO is an important enzyme for many fish species (47) that is secreted by neutrophils and monocytes during infections and plays an important role in the innate immune response as it promotes neutrophils in the blood (48, 49). It produces hypochlorous acid (HOCL) from hydrogen peroxide and chloride ion during the respiratory burst of neutrophils (50, 6).

Furthermore, it oxidizes tyrosine to the tyrosyl radical using hydrogen peroxide as an oxidizing agent, which is cytotoxic so neutrophils use to kill bacteria and other pathogens (49). MPO measurement is an indicator of neutrophil accumulation in tissues and a marker of neutrophil activity when measured in plasma (51, 52).

The non-specific immunoassay MOP (Fig. 2) recorded significant ($p<0.05$) differences after 56 days of feeding in treatment T3 compared to the rest of the treatments that did not differ significantly ($P>0.05$) from the control sample during the same period. Treatment T3 significantly ($P<0.05$) outperformed all experimental treatments after infection. Whereas, treatments T1 and T2 did not differ significantly ($P>0.05$) from the control treatment. Leya et al. (6) showed a significant increase ($P<0.05$) in MPO values when using turmeric at different concentrations as feed additives to the food of *Cirrhinus mrigala* fish after experimentally infected with *Edwardsiella tarda* bacteria, and the concentrations 1% and 1.5% gave the highest values. Al-Atbee and Al-Niaem (53) showed that the concentration of 0.5% of grape seed oil added to carp fish diets gave the highest MPO values compared with 0.2% and 1%. Ghafariarsani et al. (54) indicated a significant increase in MPO values when using thyme essential oil as feed additives for common carp.

The activity of NBT is an indicator of bactericidal activities (55), related to the respiratory blast activity that occurs

inside phagocytic cells and the production of oxides (56). When activated, phagocytes produce oxides, hydrogen peroxide, and hydroxyl radicals during the period of intense oxygen consumption, which is called a respiratory explosion (57). These roots are toxic to bacterial pathogens in fish (58). Figure (3) shows that the ratios of the NBT immunoassay for the treatments (T1 and T2) after feeding did not show significant differences (P>0.05) compared to the control sample. While after infection, the ratios of the criterion differed significantly ($p<0.05$) compared to the control treatment, and a significant increase ($p<0.05$) was observed in the percentage of NBT for T3 treatment before and after the infection. The increase in NBT values in common carp was recorded by (6) for turmeric at a concentration of 1% and 1.5%, and (53) for grape seed oil at a concentration of 0.5%, as was recorded by (2) a significant increase in MPO values with an increase in chitosan added concentrations in common carp diets.

Phagocytosis is one of the innate immune defense mechanisms (59), which is carried out by phagocytes by recognizing and eliminating invading organisms (60), Phagocytic cells protect the host's body by a process called phagocytosis of harmful organisms and dead or dying cells (61, 62). The measurement of phagocytic activity is a major indicator of the improvement of the immune response (55). The increased activity of phagocytic cells can result from natural or chemical immunostimulants that stimulate the immune system in fish (63). The percentages of phagocytic activity after feeding for the three treatments did not show a significant difference ($p>0.05$) compared with the control sample. After infection, the percentage of phagocytic activity of T1 treatment did not differ with that of the control. Which also differed significantly ($p<0.05$) from treatment T2, and the significant increase for treatment T3 ($p<0.05$) compared to other experimental treatments (Fig. 4). One of the studies that indicated an increase in phagocytic activity using plant food additives is (6) (Turmeric in common carp), (53) (Grape seed oil in common carp, (64) *Illicium verum* in *Catla catla*.

Lysozyme is a primary marker of the defense system in fish that degrades pathogens and activates the defense system and phagocytic cells through the epsonin process (65). It works by breaking down the peptidoglycan of the bacterial cell wall thus reducing infection. The high activity of this enzyme is due to the activity of various humoral factors that protect the host during pathogen invasion (66). The lysozyme activity values before and after infection in treatment T2 and T3 showed a significant ($p<0.05$) superiority over the rest of the experimental treatments, while treatment T1 did not differ significantly ($p>0.05$) compared to the control treatment, and the values of treatment T3 were significantly higher ($p < 0.05$) compared to all treatment as shown in Fig. (5). Yousefi et al. (67) reported that 200 g/kg of marjoram extract increased the activity of lysozyme enzyme. Several studies indicated that the activity of lysozyme enzyme increased by using many plants as additives in fish food, including turmeric (6) and grape seed oil (53), thyme oil (54) in common carp, star anise (*I. verum*) in *C. catla* (64)), Oregano wild thyme in Zebrafish (68). Fish require a long time to produce antibodies, so lower mortality is major evidence of immune stimulation (69, 6). Figure (6) shows the RPS survival rates of common carp after experimental infection with *S. lentus*, The third treatment T3 (1.5%) showed the highest survival rate (100%), followed by the second (1%) treatment which has survival rate of (66.67%). Yousefi *et al*. (67) indicated that the survival rate of common carp fish was significantly reduced after the challenge of infection with *Aeromonas hydrophila* of common carp fish fed on different percentages of marjoram extract compared with the control, which may result from an improvement in the antioxidant power in the fish as bacterial infection leads to stress Strong oxidizer. Similar results were reported by (2,6 and 64).

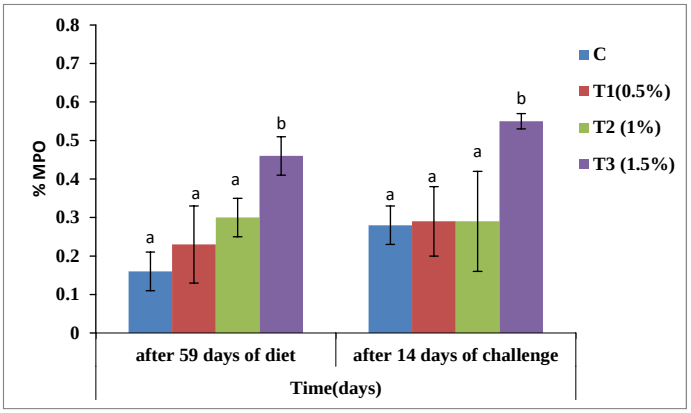

Figure (2): Myeloperoxidase activity (%) in the blood of common carp after 56 days of feeding on different percentages of marjoram aqueous extract and after 14 days of experimental infection with S. lentus.

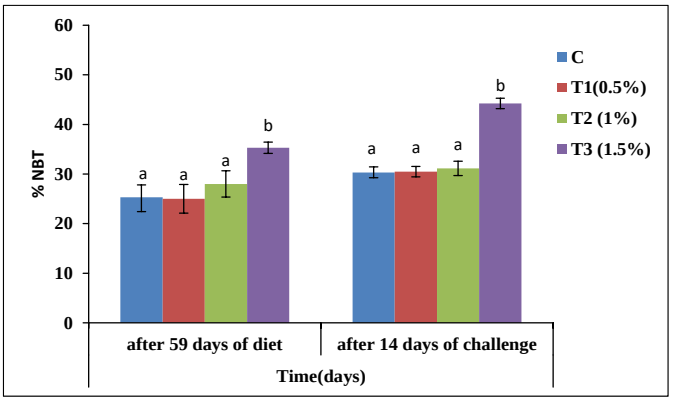

Figure (3): Effectiveness of Nitroblue tetrazolium (%) in the blood of common carp after 56 days of feeding on different percentages of marjoram aqueous extract and after 14 days of experimental infection with S. lentus

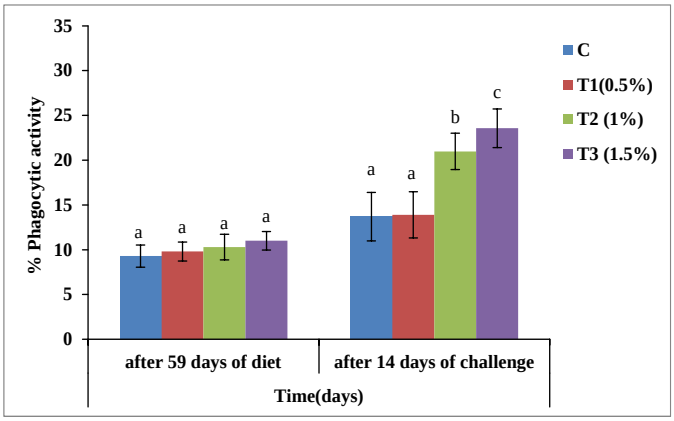

Figure (4): The effectiveness of phagocytic activity (%) in the blood of common carp after 56 days of feeding on different percentages of aqueous extract of marjoram and after 14 days of experimental infection with S. lentus.

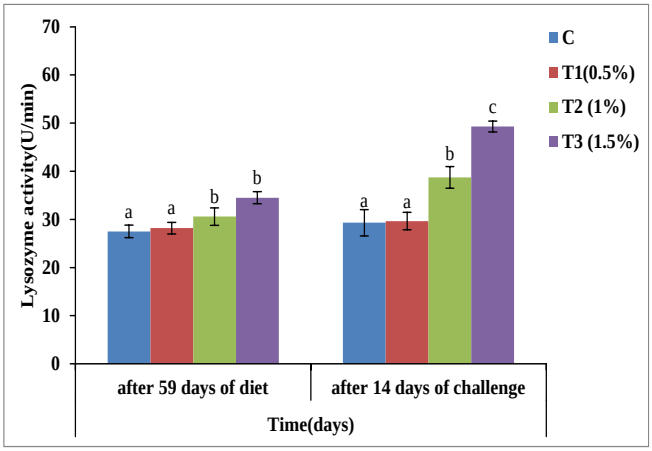

Figure (5): Lysozyme activity (units/ml) in the blood of common carp after 59 days of feeding on different percentages of aqueous extract of marjoram and after 14 days of experimental infection with S. lentus.

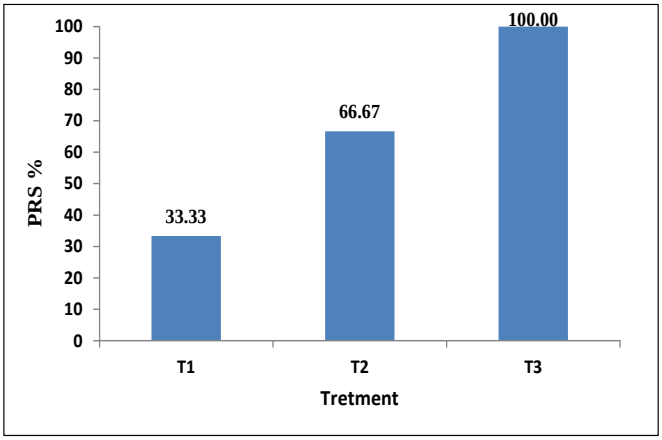

Figure (6): Relative Percentage Survival (RPS%) of common carp fish fed on rations of different percentages of aqueous extract of marjoram after 14 days of infection with S. lentus.

Conclusions

The increased activity of MPO, NBT, phagocytic activity, lysozyme activity and decreased survival rate indicated that the diet supplemented with marjoram enhances the immune response of common carp. Therefore, it is suggested to use marjoram at a rate of 1.5% as a stimulant of non-specific immunity.

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