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Efficiency of Grape (*Vitis vinifera*) Seed Oil on Nonspecific Immune Response and Histopathological Effects in Common Carp *Cyprinus carpio* Challenged with *Pseudomonas aeruginosa*

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Abstract: The present study was carried out to assess the effect of grape seed oil supplemented diet on non- specific immunity of *Cyprinus carpio* against the pathogenic bacteria *Pseudomonas aeruginosa*. Fish weights 41.69±2.25 g and total length 13.65±1.24 cm were randomly distributed into four treatments (two replications for each treatment). Fish groups were fed four dissimilar diets up to 40 days. The first group (T1) was fed with 0.2% grape seed oil, the second group (T2) was fed supplemented with 0.5% grape seed oil, the third group (T3) was supplemented with 1% grape seed oil and the forth group (T4) was served as the control group which fed basal diet without supplementing with grape seed oil. Forty-six fishes in all treatment groups were challenged intramuscularly with *P. aeruginosa* on day 41. The fishes in experimental groups were challenged intramuscularly with 0.2 ml *P. aeruginosa* at a concentration of 10^8 CFU/ml and after challenge (14 days), the different parameters were determined including nitroblue tetrazolium % (NBT), myeloperoxidases % (MPO), phagocytic activity (%) and serum lysozyme activity (U/min). The results indicated that T2 group had significant increased ($p<0.05$) in NBT activity, MPO activity, phagocytic activity and lysozyme activity compared with control group and to other treatment groups, followed by T1 and T3, respectively. In addition, T2 showed highest resistance to challenge *P. aeruginosa* compared with other groups. T3 and T4 groups showed a decreased performance in all non-specific immune parameters. There were histopathological effects in liver, showing a focal region of lymphocyte aggregation (T3), and hemorrhage into the hepatic vein with infiltration of inflammatory cells (T4). Therefore, these results indicated that 0.5% grape seed oil (T2) as additive fed could be used as prophylactic in common carp culture to enhance the protection against any possible infection by *P. aeruginosa*.

Keywords: *Cyprinus carpio*, *Pseudomonas aeroginosa*, Grape seed oil, Nonspecific immune, Histopathological effects

Introduction

The unmanaged fish culture practices and inverse environmental conditions which influence fish health leading to the economic losses in production of fishes.

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Fish diseases are causing intensive loss to the fish farmers throughout the world (Amrevuawho et al., 2014; Al-Faragi & Hassan, 2017).

Bacterial pathogens are the utmost important and in charge of severe mortalities in a broad range of fishes at various stages of growth (Swain et al., 2007; Al-Faragi, 2014). *P. aeruginosa* is found naturally in water and soil as a gram-negative opportunistic pathogen of animals and plants (Phennicie et al., 2010). It is responsible for rise mortality in hatchery of fish worldwide (Kousar et al., 2020). Disease symptom, produced by *Pseudomonas* bacteria, is a marked septicaemic hemorrhage in skin of mouth region, opercula and ventral part of the body (Phennicie et al., 2010). Antibiotics are the most strategy to bout diseases in aquaculture, and *Pseudomonas* diseases are mainly controlled by antibiotics (Ginovyan et al., 2015). However, the continuous use of antibiotics often leads to drug resistance (Tasok et al., 2017; Al-Haider et al., 2019; Al-Niaeem et al., 2020).

Controlling of diseases by the use of antibiotics has been criticized for their negative effects (Grondel et al., 1987). In order to reduce the hazard of diseases, the plane of resistance to contagion in the cultivated organisms should be increased by the use of better vaccines, immunostimulants, feeds and higher disease resistance by selective breeding (Rossi et al., 2017).

Immunostimulation is considered as a hopeful alternative treatment to antibiotics for their wide spectrum nature. Disease preventive measurement is eco-friendly and cost effectiveness. The immunostimulant is a derivative of synthetic drugs and biological agents. Amongst the biological agents are plants, and the derivatives are commonly used for disease therapy because they are versatile in nature and economical aspects. Herbs and spices such as *Cuminum cyminum* (Yilmaz et al., 2013), chitosan (Alishahi et al., 2014; Al-Mossawai & Ali, 2019) and curcumin (Tasok et al., 2017; Arslan et al., 2018) can successfully replace antibiotics in fish culture.

There are some studies which indicate to the high levels of bioactive compounds in grape seed oil (GSD) and their relationship to health benefits (Ozcan & Al Juhaimi, 2017). The main constitutive materials of grape seed oil are linoleic fatty acids, flavonoids, phenolic acids (such as gallic acid), vitamin E, ellajic acid and stilbenes (Apaydin et al., 2017). All the materials are responsible for some biological properties (Garavaglia et al., 2016) such as anti-inflammatory, antioxidant and anti-hypercholesterolemia activities (Hashemi et al., 2017). The overall objective was to evaluate the efficacy of dietary grape seed oil supplement as a resistance material against *Pseudomonas aeruginosa* in the common carp *C. carpio*.

Materials and Methods

Fish Specimens and Diet Preparation

A total of 64 live common carps, weighing 41.69±2.25 g and 13.65±1.24 cm total length, were obtained from ponds of Marine Science Centre, University of

Basrah and transported to the laboratory on 15 December 2020. The fishes were sterilized in a saturated saline solution to get rid of pathogens and ectoparasites. They were acclimated for 14 days and fed on a standard diet. Fishes were randomly distributed in eight tanks: eight fishes per tank of 30 x 40 x 60 cm. Water quality criteria were controlled by measuring daily temperature (20.6±0.23 °C), salinity (2.1±0.4 PSU), pH (8.85±0.04) and dissolved oxygen (8.8±0.4).

Fish groups were fed four dissimilar diets up to 40 days. The first group was fed with 0.2% grape seed oil, the second group was fed supplemented with 0.5% grape seed oil, the third group was supplemented with 1% grape seed oil and the fourth group, was served as the control group, fed basal diet without supplementing with grape seed oil.

The blood samples from each fish were collected by severing of the caudal peduncle to determine non-specific immune parameters.

P. aeruginosa Activation and Challenge of Fishes with *P. aeruginosa*

The bacterial culture was attended after 24 hrs by taking a swab from the stock culture of the bacteria by the loop mineral carrier and placed in a test tube containing the sterile Nutrient Broth (N.B.) feed medium. Pipes were incubated at 37 °C for 24 hrs to confirm diagnosis of the bacteria. *Pseudomonas* isolation agar (PIA) was used after autoclave sterilization and poured into Petri dishes taken from the pre-equipped 0.1 ml from the 24 hrs bacterial culture spread on the bacterial culture medium and incubated for 24 hrs at 37 °C. At the onset of growth, a gram of bacterial culture was stained and microscopically diagnosed, and examined for the growing colonies in the dish in terms of shape, colour, edge and appearance (Harley & Prescott, 2002). *P. aeruginosa* was challenged in NB 37 °C for 24 hrs, then compared with standard McFarland's tubes which were prepared according to Garvey et al. (1977). The reading was taken by the optical spectrometer with a wavelength of 600 nm. The bacterial suspension of *P. aeruginosa* was compared with the McFarland reading. Any observation of turbidity is equal when the two readings mean that the number of bacteria became 10^8/ ml.

After 40 days of the feeding period, the fishes were prepared to the challenge with 107 CFU/ ml by using intraperitoneal injection, where each fish individual received 0.2 ml of bacterial solution. Physiological saline was used in the injection for the control specimens. After infection, the animals were observed for 14 days. At this period, any external or internal symptoms on the fishes were analysed.

Non-specific Immune Parameters

The respiratory burst activity of the neutrophils was measured by nitroblue tetrazolium assay (NBT). This assay was carried out by using the reduction of NBT to formazon as a measure of superoxide anion production was done following Siwicki (1987). The lysozyme activity level was measured by using the turbidimetric assay, and the use of hen egg white lysozyme taken as standard

according to Siwicki (1987). Phagocytic activity was detected by using *Micrococcus lysodeikticus* (0.2 mg/ml) as described by Siwicki et al. (1994). According to Quade & Roth (1997), total myeloperoxidase (MPO) content present in serum was estimated.

Histopathological Examination

Specimens from fish liver were fixed in 10% neutral formalin. Paraffin sections (5 microns thick) were prepared, stained with haematoxylin and eosin (H&E) and examined under a light microscope (Humason, 1972).

Statistical Analysis

Differences among groups, in terms of non-specific immunes, were tested by one-way analysis of variance (ANOVA) at 0.05 using SPSS 20.

Result and Discussion

Non-specific Immune Parameters

Immune system stimulation of fishes through novel drugs, especially from plant sources, is of great interest for commercial aquaculture (Tasok et al., 2017).

Myeloperoxidase activity of the control group and grape seed oil supplemented groups is presented in Table 1. MPO activity after 40 days dietary feeding of grape seed oil was significantly ($p<0.05$) higher in grape seed oil groups compared to control group. After challenge, 0.5% grape seed oil group (T2) registered the highest MPO activity (2.46%) which showed significant difference than T1, T3 and T4 (Table 2).

MPO is a heme-containing peroxidase expressed mainly in neutrophils and to a lesser degree in monocytes. In the presence of hydrogen peroxide and halides, MPO catalyzes the formation of reactive oxygen intermediates, including hypochlorous acid (HOCl). The MPO/HOCl system plays an important role in microbial killing by neutrophils. In addition, MPO has been demonstrated to be a local mediator of tissue damage and the resulting inflammation in various inflammatory diseases (Sahu et al., 2007). It utilizes hydrogen peroxide during the respiratory burst to produce hypochlorous acid (Sahoo et al., 2005). In the present finding, the treatment groups showed higher MPO activity (except T3) compared to the control group. A similar result was reported in case of garlic (Sahu et al., 2007) and *Curcuma longa* (Tasok et al., 2017).

Respiratory burst activity (NBA) in *C. carpio* neutrophils indicated significant ($p<0.05$) increase in all grape seed oil groups compared to control group after 40 days feeding of grape seed oil (Table 1). There was a significant ($p<0.05$) difference between T1 and T3. After challenge (Table 2), NBA increased significantly in all grape seed oil groups in comparison to control group. T2 showed the highest level of NBT which was significantly different than in other groups (T1, T3 and T4). The phagocytic activity (%) was found to be significantly ($p<0.05$) higher after 40 days dietary of 0.5% grape seed oil compared to control, and not significant ($p>0.05$) in dietary of 0.2% and 1% fed in grape seed oil (Table 1). After

challenge with *P. aeruginosa*, T2 group revealed significant difference in comparison to T1, T3 and T4, while T3 showed a significant decrease in phagocytic activity compared to the other treatments (Table 2). Phagocytosis is a critical biological activity through which the host can protect itself from infectious and non-infectious environmental particles and remove unwanted host cells in order to maintain tissue homeostasis (Øverland et al., 2010; Mu et al., 2019).

The possible reason for these results is that low concentrations of food additives contain limited quantities of active substances, and the fish ability to benefit from them is limited, as increasing their concentration will give negative results due to the energy spent by the fish to get rid of the increased concentrations (Olsen et al. 2001; Ringø et al., 2010; Akrami et al., 2013).

NBT activity is typically associated with respiratory burst activity, characterized by intracellular localization of super oxide anion in phagocytes (Sahoo et al., 2005). Phagocytes, after activation, generate super oxides and other reactive compounds (i.e. hydrogen peroxide and hydroxyl radical) during the period of intense oxygen consumption called respiratory burst. These oxygen reactive radicals are considered toxic for bacterial fish pathogens (Siwicki, 1987). In the present study, the highest activity of NBT was observed in T2 group which fed with 0.5% of grape seed oil that was beneficial in protecting them from the disease-causing organism (*P. aeruginosa*). Other immunostimulants such as garlic (Sahu et al., 2007), Curcumin (Tasok et al., 2017), are also known to stimulate phagocytes in fishes.

The serum lysozyme activity of *C. carpio* after 40 days feeding trail on grape seed oil and after challenge with *P. aeruginosa* is shown in Table 1. The results exhibited the highest lysozyme activity (35.3 U/ml) in T2 fish group. There was significant ($p<0.05$) difference between T2 and control group. T1 showed no significant ($p>0.05$) difference compared to T3 and control groups.

The serum lysozyme activity after challenge with *P. aeruginosa*, T3 group revealed significant ($p<0.05$) difference in comparison to control group (T4) and also significant ($p<0.05$) difference than T1 and T3. In addition, T3 showed a significant decrease compared to the other treatments (Table 2). It is well known that lysozyme is a primary marker of fish defense system, which causes lysis of pathogens, activation of the complement system and phagocytes by acting as opsonin (Magnadóttir, 2006). Lysozyme is an enzyme that breaks down the peptidoglycan (β-1, 4-glycosidic linkages) of the bacterial cell wall, thereby controlling the infection. An increase in the lysozyme activity suggests elevation of various humoral factors that protect the host during pathogen invasion (Harikrishnan et al., 2010). It was also previously reported that serum lysozyme activity could be enhanced through dietary administration by various herbs in Chinese sucker *Myxocyprinus asiaticus* (Zhang et al., 2009) and *Labeo rohita* (Das et al., 2015) treated with tulsi (*Ocimum sanctum*) extracts; in yellow crocker *Pseudosciaena crocea* supplemented with Astragalus root (Jian & Wu, 2003) and *L. rohita* fed with andrographolide (Basha et al., 2013).

Table1: The nonspecific immune response activities in in common carp fed with diets supplemented with different doses of grape seed oil after 40 days.

Nonspecific immune response	T1 (0.2%)	T2 (0.5%)	T3 (1%)	T4 (Control)
MOP activity (%)	0.23±0.05[a]	0.46±0.05[b]	0.3±0.1[a]	0.16±0.05[c]
NBT activity (%)	25±2.64[a]	43.3±1.15[b]	28.3±2.88[c]	22.3±2.51[a]
Phagocytic activity (%)	7.3±2.08[a]	16.6±1.52[b]	12.3±1.52[c]	7±2.64[a]
Serum lysozyme activity (U/min)	25.3±2.51[a]	35.3±2.3[b]	27.6±2.3[a]	23±2.0[a]

Different letters in the same row are significantly different ($p<0.05$). MOP: Myeloperoxidase NBT: Nitroblue tetrazolium assay.

Table 2: The nonspecific immune response activities in common carp after 14 days of challenge with *P. aeruginosa*.

Nonspecific immune response	T1 (0.2%)	T2 (0.5%)	T3 (1%)	T4 (Control)
MOP activity (%)	0.66±0.20[a]	2.46±0.11[b]	0.12±0.05[c]	0.10±0. 02[d]
NBT activity (%)	37.66±1.52[a]	79.66±2.08[b]	15.03±1.42[c]	18.33±2.08[d]
Phagocytic activity (%)	34.3±2.08[a]	77.6±2.30[b]	5.6±1.01[d]	5.5±1.21[d]

Different letters in the same row are significantly different ($p<0.05$). MOP: Myeloperoxidase NBT: Nitroblue tetrazolium assay.

Histopathological Changes

Some pathological signs were appeared in the tissues among different groups. These changes included hemorrhage into the hepatic vein, lymphocyte aggregation and inflammatory cells (Huizinga et al., 1979).

Plate 1 showed histological sections of liver of control fishes, before their challenge with *P. aeruginosa*, showing the normal hepatic tissue, hepatic cords and liver sinusoid (Plate 1A), while Plates 1B and C indicated that fishes fed diet containing 0.2% and 0.5% grape seed oil, showed no effect on hepatocytes. Plate 1D showed that the pathological changes represented hemorrhage into the hepatic vein with infiltration of inflammatory cells (liver of control fishes, after 14 days of challenge with *P. aeruginosa*). In Plate 1E, liver of fishes, fed diet containing 1% grape seed oil, showed a focal region of lymphocyte aggregation migrating from dilated blood vessels to degenerate hepatocytes.

The reason of occurring these pathological changes is due to high intensity of *P. aeruginosa* which obstruct metabolism in the liver, especially in the treatment T3, because of the energy exchange by the fishes to get rid of the increase in grape seed oil (Akrami et al., 2013). The histopathological changes in the current study are similar to those of Magdy et al. (2014) on African catfish *Clarias gariepinus*.

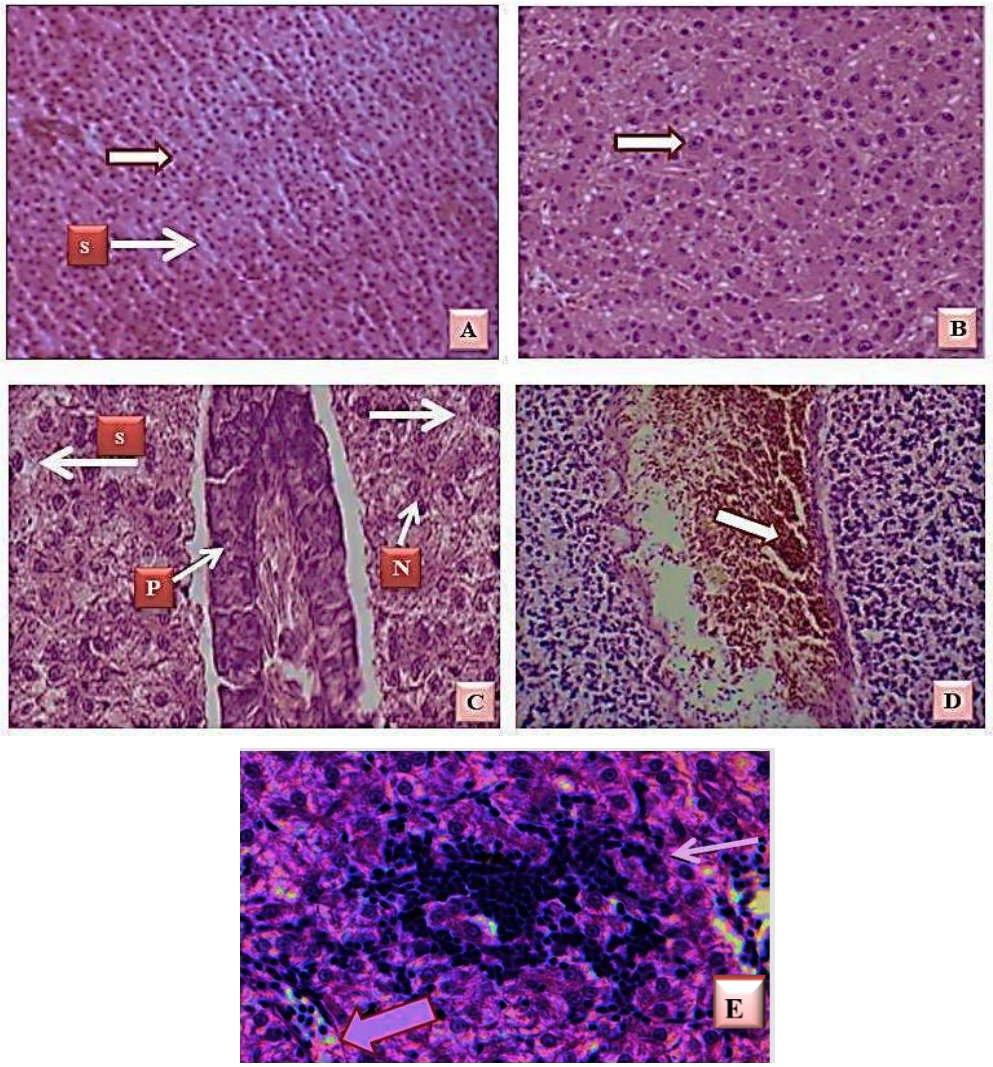

Plate 1: Histological sections of liver. A: Liver of control fishes, before challenge with *P. aeruginosa*, showing the normal hepatic tissue, hepatic cords (white thick arrow) and liver sinusoid (S) (200X, H&E stain). B: Fishes fed diet containing 0.2% grape seed oil, the liver of fishes showing hepatic cords (white thick arrow) with no effect on hepatocytes (400X, H&E stain). C: Fishes fed diet containing 0.5% grape seed oil, the liver of fishes showing sinusoid (S), pancreas (P) and nucleus (N) with no effect on hepatocytes (400X, H&E stain). D: Liver of control fishes, after 14 days of challenge with *P. aeruginosa*, showing the liver with hemorrhage into the hepatic vein with infiltration of inflammatory cells (arrow) (400X, H&E stain) and E: Fishes fed diet containing 1% grape seed oil, the liver of fishes showing a focal region of lymphocyte aggregation (thin arrow) migrating from dilated blood vessels (thick arrow) to degenerated hepatocytes (400X, H&E stain).

Conclusion

The grape seed oil has beneficial effects, which included the protection against oxidative damage in cells, and anti-inflammatory effects in fishes. Non-specific immune responses were elevated in common carp. Thus, use of grape seed oil for fries of this fish at 0.5% as nonspecific immune response promoter is suggested.

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