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Effect of Three Plant Oils on *Aeromonas hydrophila* Infection, Immune-Related Renal Gene Expression, and Serum Biochemical Parameters in the Common Carp

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ABSTRACT

Medicinal plants can be cost-effective, more productive, and alternative anti-bacterial agents in aquaculture, exhibiting many biological effects as anti-stress action and immune stimulation against various diseases. The present study aimed to evaluate the effect of three types of plant oils, namely mint, chamomile, and ginger on some serum biochemical parameters, *Aeromonas hydrophila* infection, as well as immune performance and mRNA levels of some renal immune-related genes (*il-1β, il-8, il-10,* and *tnf-α*) in common carp. We used for this purpose 120 fish with an average weight of 250 ± 4 g. Fish were fed on an experimental diet of 5% of the weight divided into two meals per day. The experiment continued for 120 days with adaptation. The results showed that mint oil had a superior effect on the serum total protein, ginger oil treatment induced the highest serum alkaline phosphatase activity, and chamomile oil treatment induced the highest serum alanine aminotransferase activity. Both ginger and mint oil treatments recorded the highest serum aspartate aminotransferase activity. The mint treatment proved effective at treating experimentally-induced *Aeromonas hydrophila* infection, reducing the infection rate to 0%. Mint oil treatment caused a *significant elevation* in the expression of renal *il-8, il-10,* and *tnf-α*mRNA, chamomile oil treatment caused a significant elevation in the expression of renal *il-8* and *il-10* mRNA, and ginger oil treatment caused a significant change in the expression of renal *il-1β, il-8,* and *tnf-α.* The results showed that these three oils improved production performance and enhanced the expression of some immune-related genes against bacterial infections.

INTRODUCTION

Aquaculture growth is often associated with the intensification of agriculture, which leads to crowding and poor water quality, facilitating the spread of pathogens and increasing disease outbreaks and fatalities **(Bondad *et al.*, 2005)**. In order to avoid economic losses related to health deficiencies, veterinary drugs are widely used in fish farming to prevent and treat disease **(Rico *et al.*, 2013)**. Consequently, because of the many disadvantages of synthetic medicinal drugs used to treat disease, there is a growing

need to develop alternative strategies for managing aquatic diseases **(Ashley *et al.*, 2007)**. Medicinal plants have been used in aquaculture, not only as chemotherapy, but also as feed additives, due to their wide range of nutrients and chemical compounds **(Chang, 2000)**. Medicinal plants also act as a catalyst for the cellular and humoral immune response, which was evident by the rise in the immune parameters in the blood of fish **(Awad and Amani, 2017)**.

Aeromonas hydrophila is the main cause of toxemia in fish, including the common carp *Cyprinus carpio* **(Chen *et al.*, 2018)**. Common carp was recently found, along with several other types of freshwater fish, such as *Oreochromis niloticus, Carassius auratus, Pangasianodon hypophthalmus*, and others, to be a type suitable for breeding in high salinity conditions **(Iffat *et al.*, 2019)**.

Three types of plant oils that have a therapeutic history have been chosen for this study. Mint contains a volatile oil of concentration 1.5%, which is responsible for the therapeutic effect of the plant, as it works as a natural antibiotic **(Mohaddese and Nastaran, 2014)**, chamomile that has antibacterial, antipyretic, ulcerative, antifungal, and antioxidant properties **(Stuart, 2014)**, and ginger that is effective at treating a range of bacterial, viral, fungal, and parasitic diseases **(Kim *et al.*, 2007)**. The present study aimed to assess the effect of these three types of plant oils (mint, chamomile, and ginger) on the expression of some immune related genes in kidney, like *il-1β, il-8, il-10*, and *tnf-α*, some serum biochemical parameters, and *Aeromonas hydrophila* infection in common carp *Cyprinus carpio* L. reared in floating cages.

MATERIALS AND METHODS

Zone of study and fish collection

The study was conducted from August 2019 to December 2019. Initially, an acclimatization period of 8 days was included in floating cages on the Euphrates River, Nasiriyah, Iraq. The sample consisted of 150 common carps that fed at 3% by weight twice a day. Subsequently, 120 fish with an average weight of 250 ±4 g were selected, and 10 fish per replicate were randomly distributed in four groups in three replications.

Manufacturing of diets

The feeds were manufactured locally at the site of the experiment, as they were prepared from forage materials available in the local markets. Separately, the mint, chamomile and ginger oils were added at 5% concentration to the diet, as recommended in a previous study **(Dairiki *et al.*, 2013)**. The average weight of the fish was measured every two weeks. The feeding method involved the use of submersible pots. The control group lacked additives.

Blood samples

Fish blood (2-3 ml) was collected by direct puncture of the tail vein using a syringe without an anticoagulant. The blood was placed in a sterile 10 ml test tube, after which it was centrifuged for 5 min at a speed of 3000 rpm, and the serum was collected by a special pipette. Then, the required biochemical parameters were measured in serum.

Serum parameters

Serum level of total protein (TP), and the activities of alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) were estimated using Gesan Chem-200 platform (Gesan Production SRL, Italy) according to manufacturer's instructions.

Bacterial isolation and experimental infection

Aeromonas hydrophila bacteria were isolated from infected fish with Motile Aeromonas Septicemia (MAS) disease at a fish farm on the Euphrates, about 20 km from the site of the study. After 105 days from the start of administration of the dietary supplement oils, bacterial colonies were picked and suspended in physiological solution, to prepare a dose of 1 ml containing around $1x10^7$ bacterial cells, and injected under the pectoral fin of each fish.

Total RNA isolation, RT-PCR, and real-time PCR assay

RNA was extracted from kidney tissue using an RNA-spin Total RNA Isolation Kit (iNtRON Biotech., South Korea); because most of the rapidly evolving immune genes were widely expressed in the head of kidney, which significantly affected the activation of immune response. The reverse transcription PCR (RT-PCR) cDNA synthesis was carried out using HiSenScript RH (-) RT PreMix Kit (iNtRON Biotech., South Korea). The RT-PCR conditions were one thermocycle of 50°C for 45 minutes, and 85°C for 10 minutes.

Real-time PCR was performed by using the Real-Time PCR Mx3000P system (Stratagene technologies, Agilent, USA). The PCR components were prepared by 10 Mm primers (Table 1), 10 µl of Real MODTM Green SF 2X qPCR mix (iNtRON Biotech., South Korea) and 100 ng cDNA strand and nuclease-free water up to final volume 20 µl. The PCR conditions were 95°C for 10 minutes, 40 thermocycles of 95°C for 15 seconds, 60°C for 1 minute. Subsequently, a dissociation curve was applied with one cycle at 95°C for 1.0 minute, 55°C for 30 seconds, and 95°C for 30 seconds, and the reaction conditions were adjusted according to the manufacturer's instructions.

Table (1): Sequence of primers used for qPCR experiments for *il-1β, il-8, il-10, tnf-α,* and *β-actin* genes.

Gene	Primer sequence (5′-3′)	Reference
il-1β	F: ACATTGCCAACCTCATCATCG R: TTGAGCAGGTCCTTGTCCTTG	**(Qiang *et al*., 2016)**
il-8	F: AGAATGTCAGCCAGCCTTGT R: TCTCAGACTCATCCCCTCAGT	**(Ming *et al*., 2013)**
il-10	F: CG CAGTGCAGAAGAGTCGAC R: CCCGCTTGAGATCCTGAAATAT	**(Chakrabarti *et al*., 2014)**
Tnf-α	F: CCAGGCTTTCACTTCAGG R: GCCATAGGAATCGGAGTAG	**(Chakrabarti *et al*., 2014)**
β-actin	F: AGACCACCTTCAACTCCATCATG R: CCGATCCAGACAGAGTATTTACG	**(Chakrabarti *et al.*, 2014)**

interleukin 1β (il-1β), interleukin 8 (il-8), interleukin 10 (il-10), tumor necrosis factor alpha (tnf-α), internal reference gene (*β -actin*, house-keeping gene).

Statistical Analysis

The experiment was designed according to Complete Random Design (CRD), and statistical analysis was conducted using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). The significant differences between the parameters were tested **(Duncan, 1955)**. RT-PCR results for target genes and housekeeping β-Actin gene were analyzed by measuring the relative amount of gene expression and fold change, as described by **Livak (2001)**.

RESULTS AND DISCUSSION

The levels of total protein in serum showed significant differences ($P \leq 0.05$) between the experimental treatments, the dietary mint supplements had the highest total protein levels at 4.04 mg/100 ml, while there were no significant differences ($P > 0.05$) in the levels of serum total protein between chamomile and ginger treated groups, 3.60 mg/100 ml and 3.50 mg/100 ml, respectively. The control group recorded the lowest mean of serum total protein concentration, 3.13 mg/100 ml (Fig.1).

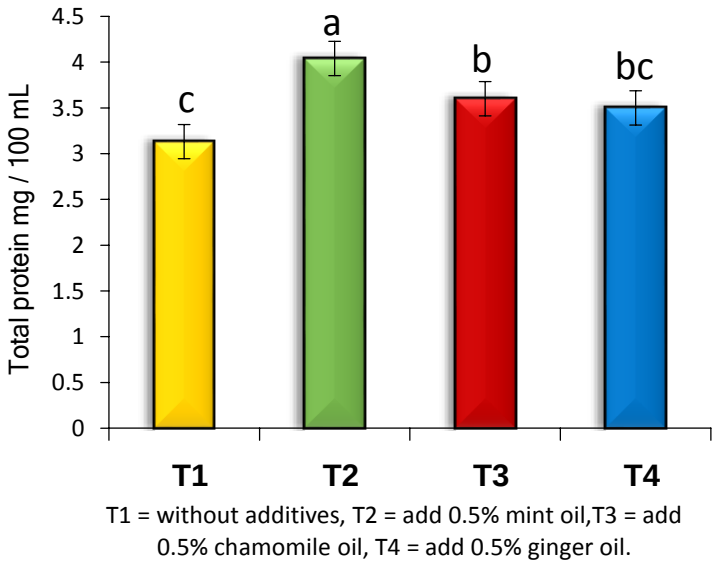

Fig. 1. Serum total protein concentration in the control and treated groups.

The mint treatment was found to be superior to the rest of the treatments, in terms of the levels of total protein. This may be because of the phenolic compounds in peppermint oil, which have a positive effect on the digestibility of food, which improves the growth rate and the general health of the fish **(Giannenas *et al.*, 2003)**. As for chamomile supplement, it may contribute to increase the efficiency of the digestive system, because of the soothing effects of chamomile on the stomach **(Shoara *et al.*, 2015)**.

The activities of ALP, ALT, and AST in fish serum showed significant differences ($P \leq 0.05$) in the oil dietary treated groups (mint, chamomile, and ginger) compared to the control group. The group treated with ginger dietary supplement recorded the highest ALP activity at 90.66 U/L, followed by mint and chamomile treated groups at 89.33 U/L and 87.67 U/L, respectively, and the control group had the lowest ALP activity at 83.0 U/L (Fig. 2). The group treated with chamomile dietary supplement showed the highest ALT activity at 14.66 U/L, followed by mint and ginger treated groups at 13.66 U/L and 13.50 U/L, respectively, while the control group recorded the lowest ALT activity at 10.67 U/L (Fig. 2). There was no significant difference ($P>0.05$) between the ginger and mint dietary treatments, as the treatment with ginger resulted in the highest activity of AST at 93.33 U/L, followed by the mint treatment at 92.33 U/L, and chamomile treatment at 90.0 U/L, while the control group recorded the lowest activity of the AST at 85.67 U/L (Fig.2).

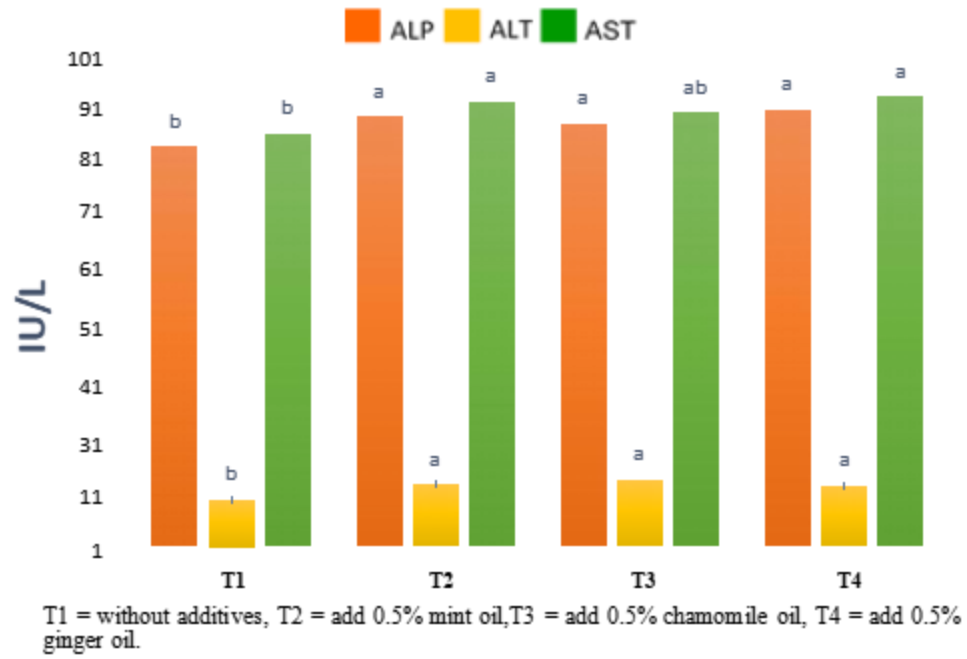

Fig. 2. Serum ALP, ALT, and AST activities in the control and treated groups

The somewhat significant increase in the serum ALT, AST, and ALP activities in oil-treated fish indicated that the oil supplements may negatively affect the liver function. Despite the increase in the activity of enzymes in this study, we did not observe any negative signs in the activity of the fish or its health deterioration.

After 7 days of infecting fish with *Aeromonas hydrophila* through injection, the following moderate-intensity symptoms of MAS appeared: lethargy, swimming near the surface, cracking of the caudal fin with blood ulcers on the body, abundant mucus surrounding the body, loss of appetite, and fragility of kidneys, and change in the color of the spleen to dark red. The results indicated that the fish treated with mint were not infected with MAS, as the infection rate was 0%, while the infection rate for fish treated with chamomile and ginger were 20%, and in the control group, the infection rate reached 100%.

The study showed a significant increase ($P \leq 0.05$) in the expression of *il-1β* mRNA in the group treated with ginger dietary supplement, with a 28.33-fold change compared to the control group, while expression of *il-1β* mRNA was not significantly affected in the groups treated with mint and chamomile supplements compared to the control group (Fig.3). The three treatments: chamomile, ginger, and mint, showed a significant increase ($P \leq 0.05$) in *il-8* gene expression compared to the control group. Chamomile supplement recorded a 4.55-fold change of *il-8* mRNA expression compared to the control group (Fig. 3). The results indicated that mint treatment caused a significant increase ($P \leq 0.05$) in *il-10* mRNA expression (10.58-fold) compared to the control group.

The chamomile treatment caused a significant increase (P≤0.05) in *il-10* gene expression (8.03-fold) compared to the control group. However, there were no significant differences in *il-10* gene expression between the ginger and control (Fig.3). Mint treatment led to a significant elevation in the gene expression level of *tnf-α* (P≤0.05) compared to the control (4.96-fold), and ginger treatment led to a significant elevation in *tnf-α* gene expression level (P≤0.05) compared to the control (2.84-fold), while there were no significant differences in gene expression of *tnf-α* between chamomile treated group and control (Fig. 3).

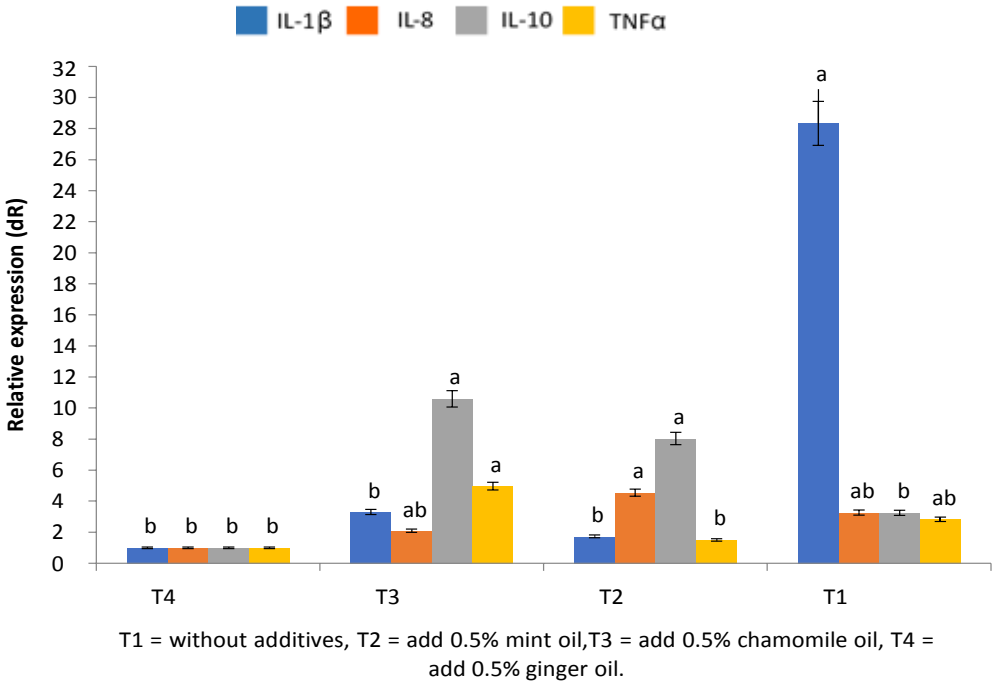

Fig. 3. gene expression of *il-1β, il-8, il-10*, and *tnf-α* genes in kidney of the control and treated groups.

However, the effectiveness of mint oil in fighting *Aeromonas hydrophila* infection and enhancing immunity in fish was evident, and this may be due to the fact that mint compounds, including menthol, exhibit strong anti-bacterial activity against *staphylococcus*, *staphylococcus aureus*, and *staphylococcus cutaneous*, and especially against *Escherichia coli* **(Mimica *et al.* 2003; Mahboubi *et al.*, 2008)**. Chamomile oil has been found to be effective against *Aeromonas hydrophila* infection, and enhances fish immunity. This may be due to the antioxidant substance chamazulene in chamomile, which has strong anti-bacterial activity against *Escherichia coli* and other bacteria **(Grzanna *et al.*, 2005)**. In addition, chamomile showed a good wound-healing effect, as well as an anti-inflammatory effect. The results also indicated the effectiveness of ginger oil in fighting *Aeromonas hydrophila* infection, and this may be due to the fact that ginger contains compounds, including zingiberene, that have shown good efficacy in

resisting infectious diseases by enhancing specific and non-specific immune mechanisms **(Harikrishnan *et al.*, 2011)**. Ginger has also shown to be effective in controlling a range of bacterial, viral, fungal, and parasitic diseases **(Kim *et al.*, 2007)**.

CONCLUSION

Although the plant oils of mint, chamomile, and ginger may induce some tissue damage in fish by the elevation in serum ALT, AST, and ALP activities, their effectiveness in combating *Aeromonas hydrophila* infection and positively raising the level of gene expression of *il-1β, il-8, il-10, tnf-α* have proven in the present study.

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