Comparative effects of garlic, black seed, and black caraway encapsulated essential oils powder on the histopathology of rainbow trout (*Oncorhynchus mykiss*) gills, kidneys, and intestines

Pourmand Z.[1]; Kazempoor R.[2*]; Kakoolaki Sh.[3]; Khajerahimi A.[2]; Ghorbanzadeh A.[4]

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Abstract

Natural compounds derived from plants possess antimicrobial, antifungal, and anti-inflammatory properties, enhancing the immune system, promoting growth, and reducing stress in fish. This study investigated the effects of dietary supplementation with encapsulated powders of essential oils from garlic, black seed, and black caraway on the vital organs of rainbow trout (*Oncorhynchus mykiss*), specifically the kidneys, gills, and intestines. Due to the natural instability of essential oils, encapsulation was employed to preserve their therapeutic properties. Initial extraction of pure essential oils was followed by identification of their components using gas chromatography-mass spectrometry (GC-MS) and subsequent encapsulation using a fluidized bed dryer for improved stability. Rainbow trout were divided into experimental groups: a control group fed a basal diet, a black seed group (basal diet + black seed), a garlic group (basal diet + garlic), a black caraway group (basal diet + black caraway), and a combined group (basal diet + a combination of the three essential oils). The fish were fed these diets for eight weeks. Histopathological examinations revealed significant damage in the gills of the black seed and combined oil groups, characterized by hyperplasia and inflammation. In the kidneys, the combined group exhibited pronounced proteinuria and hemorrhage, while increased villi length and mucosal cell hyperplasia were noted in the intestines of this group. Results indicated that garlic oil had the most positive effect on tissue health, showing the least damage. Thus, garlic oil is recommended as a dietary supplement for improving the health of rainbow trout in sustainable aquaculture practices.

Keywords: Essential Oils, Histopathology, Rainbow Trout, Encapsulation, Dietary Supplementation.

1-Resident in Aquatic Animal Health and Diseases, Science and Research Branch, Islamic Azad University, Tehran, Iran
2-Department of Aquatic Animal Health and Diseases, Science and Research Branch, Islamic Azad University, Tehran, Iran
3-Department of Aquatic Animal Health and Diseases, Agricultural Research, Education and Extension Organization (AREEO), Iranian Fisheries Science Research Institute, Tehran. Iran
4-Assistant Professor of Ichthyo Pathobiologist, Faculty of Veterinary Sciences, Department of Aquatic Healt and Diseases, Science and Research Branch, Islamic Azad University, Tehran, Iran
Corresponding author's Email: rkbs_kh@yahoo.com

Introduction

Aquatic animals are an excellent source of food that provide the body with plenty of protein, essential amino acids, and other important nutrients. Regularly eating aquatic life and fish can help meet the body's needs for amino acids and certain key minerals, which is crucial for overall health and strong development (Mansour *et al*., 2022). Additionally, fish farming in countries with abundant water resources can generate export income for those nations. Despite the promising future of aquaculture, there are also challenges such as water quality issues, disease outbreaks, nutrition problems, and sometimes stressful environmental conditions (Føre *et al*., 2018; Suzuki, 2021). Water quality is one of the most critical factors in aquaculture. Water contaminated with organic matter, pollutants, toxic substances, or with an unsuitable pH can cause serious problems for aquatic animals. Poor water quality can lead to disease, breathing issues, inadequate growth, and even the death of aquatic creatures (Verma *et al*., 2022). Aquatic animals are exposed to many different diseases and infections. These illnesses can spread through the water, food, infectious agents, or living carriers. Disinfectants, vaccines, continuous monitoring, and proper healthcare are essential to address disease-related concerns (Assefa and Abunna, 2018). Providing a balanced and appropriate nutrition for aquatic animals is also extremely important. Nutrient deficiency or excess, improper amino acid ratios, and imbalance of vitamins and minerals can result in poor growth, weakened immune systems, and nutrition-related diseases (Awuchi *et al*., 2020). Environmental factors like temperature, light, oxygen, and water flow also affect the life of aquatic animals. Unfavorable conditions can cause stress, reduced growth, increased disease, and even the death of aquatic creatures. Solutions have been defined and sometimes implemented to address each of these challenges, but some have not been entirely successful (Reid *et al*., 2019).

The challenge of using antibiotics in aquaculture is closely linked to the overuse and improper prescription of antimicrobial agents in the rearing of aquatic animals. This issue is widely recognized as a serious concern within the aquaculture sector (Santos and Ramos, 2018). One of the most suitable methods for preventing disease in fish is to enhance immunity and strengthen the body's defense mechanisms. In fish, the response to pathogens involves both innate and adaptive immunity, but the activation of the innate immune response is more consequential and relevant compared to the adaptive immune response (Kordon *et al*., 2018; Alavinejad *et al*., 2022). Therefore, the use of immune stimulants is an accepted and promising approach for bolstering and improving the immune system in aquaculture. Immune stimulants are natural or synthetic biological compounds that can activate the immune system. Most immune stimulants have a strong influence on the non-specific (innate) immune system, which is highly beneficial and effective for fish, and they may also have a positive effect on antibody production (Stratev *et al*., 2018; Vijayaram *et al*., 2022). Additionally, these stimulants can be easily administered to smaller fish. Using immune stimulants prior to the anticipated outbreak of a disease can increase immunity and disease resistance. While stimulating immunity is less effective compared to vaccination and does not provide long-term immunity or the rapid therapeutic effect of chemical drugs, it is a preferable approach due to the lack of harmful side effects, broader effectiveness, and no pathogen resistance (Kumar *et al*., 2023). Many immune stimulants have been employed in aquaculture, including synthetic, biological, animal-derived, and plant-based compounds. Plant-based immune stimulants are particularly promising because they are cost-effective, readily available, and environmentally friendly, without leaving any harmful residues or causing pathogen resistance (Naderi *et al*., 2022; Mariappan *et al*., 2023).

Natural oils extracted from plants have useful properties, like killing microbes, reducing inflammation, and acting as antioxidants and disinfectants (D'agostino *et al*., 2019). In fish farming, these natural plant oils can be used as a natural way to boost the immune system of fish and improve their growth (Souza *et al*., 2019). The plant oils can have different effects on fish. Some have antimicrobial and antifungal effects, which can help fish resist infections and stay healthy (Sutili *et al*., 2018). Others have anti-inflammatory effects that can make fish better able to handle changes in their environment like temperature, salt levels, and chemicals (Majolo *et al*., 2018). Some plant oils also have calming effects that can help fish cope with unfavorable conditions. Using these different plant oils can lead to better growth and performance in farmed fish (Abdel-Latif *et al*., 2020; Kazempoor *et al*., 2022). Several studies have found that oils from garlic, black seed, and black caraway have beneficial antimicrobial, anti-stress, and immune-boosting effects (Roohi *et al*., 2017; Sutili *et al*., 2018; Souza *et al*., 2019; Adineh *et al*., 2020; Rouf *et al*., 2020; Yousefi *et al*., 2021; Kumar *et al*., 2023). Given the proven benefits of these plant oils, this study will look at how adding powders of garlic, black seed, and black caraway oils to the diet affects the kidneys, gills, and livers of rainbow trout (*Oncorhynchus mykiss*).

Materials and methods

Preparation of essential oils, relevant evaluations, and determination of essential oil compositions

The essential oils of interest were purchased from the Tabib Daru (Iran, May 2022) pharmaceutical company. The effective phytochemical compositions were determined using the GC-MS (gas chromatography-mass spectrometry) method, in which helium gas was used as the carrier, and a 1 μL sample of the essential oil was injected into the instrument's chamber for analysis (Al-Rubaye *et al*., 2017). The GC/MS analysis was performed on an Agilent GC 7890A instrument equipped with an HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) coupled with an EI MS 5975 C model (Agilent Company, USA). The oven temperature program, carrier gas flow rate, split ratio, and injector temperature were set as specified. The essential oil components were identified by comparing their retention times and mass spectral fragmentation patterns with those stored in the computer libraries of Wiley 7n.1 and NIST (National Institute of Standards and Technology).

Determination of antibacterial activity

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the encapsulated essential oils against three fish pathogenic bacteria - Yersinia rookeri, Lactobacillus Garvey, and Streptococcus iniiae - were investigated (Lee *et al*., 2009). The broth dilution method was used for this evaluation. Serial dilutions of the essential oils in DMSO were prepared, ranging from 0.125% to 10%. The bacterial suspensions were added to the dilution tubes, and the microplates were incubated for 24 hours at 37°C. The concentration of the most diluted well that showed no visible growth was considered the MIC. To determine the MBC, samples from the wells with no visible growth were cultured on TSA media, and the lowest concentration that showed no bacterial growth was considered the MBC. Additionally, the essential oil extracts at a 20% concentration were tested using the agar disk diffusion method.

Preparation of essential oil capsules" section

Garlic, black seed, and black caraway essential oils were encapsulated using alginate. Preparing an alginate solution (3%) in water, then adding essential oils to the alginate solution and creating an emulsion with the help of an emulsifier (such as Tween 80), then adding this emulsion drop by drop to a 2% calcium chloride solution ($CaCl_2$) to create microcapsules, separating the microcapsules After forming and finally drying according to the Floyd method, it is dry.

Classification of fish and their feeding with encapsulated essential oils

This study was conducted in the fall of 2023 at a coldwater fish farm in Firoozkooh, Iran. Two hundred rainbow trout with an initial average weight of 10±225 grams were randomly divided into 5 groups, with each group consisting of 40 fish. The fish were reared in 5 ponds with similar water quality parameters, including a temperature range of 9.8 to 13°C, pH between 6.5 to 7.3, and dissolved oxygen concentration of 6 to 7 mg/L. The experimental groups included a control group fed a basal diet, a black seed group (basal diet + black seed), a garlic group (basal diet + garlic), a black caraway group (basal diet + black caraway), and a black seed, garlic, and black caraway group (basal diet + a mix of the three essential oils). The essential oils were added to the feed pellets at a concentration of 3 g per 15 kg of feed. During the 8-week feeding trial, the fish were manually fed twice a day at a rate of 2% of their body weight.

Histological analysis of gill, kidney, and intestine tissues

Tissue samples of gill, kidney, and intestine were collected from the control group and the experimental groups (garlic, black seed, black caraway, and a combination) at the end of the experimental period. The fish were euthanized, and the tissues were carefully dissected and harvested. The collected tissue samples were immediately fixed in 10% neutral buffered formalin solution, and the fixation process was carried out at room temperature for at least 24 hours. The fixed tissue samples were then dehydrated through a graded series of ethanol solutions, cleared in xylene, and infiltrated and embedded in paraffin. The paraffin-embedded tissue blocks were sectioned using a rotary microtome, and thin sections (4-6 micrometers thick) were mounted on clean microscope slides. The tissue sections were stained using standard histological techniques, such as hematoxylin and eosin (HandE) staining. The stained tissue sections were examined under a light microscope, and detailed observations and evaluations of the histological changes in the gill, kidney, and intestine tissues were recorded for each experimental group. Micrographs were taken to document the observed histological features in the tissue sections, and the collected microscopic data were analyzed and interpreted to determine the effects of the different treatments on the gill, kidney, and intestine tissues (Mokhtar, 2021).

Essential oil composition

Table 1 presents the detailed chemical composition of garlic essential oil as determined by GC-MS analysis. The major constituents are allyl sulfide (11.12%), allyl disulfide (46.15%), diallyl trisulfide (31.52%), and diallyl tetrasulphide (10.60%), which together make up the majority of the oil. Smaller amounts of other sulfur-containing compounds were also detected.

Table 1: Garlic essential 0il composition by GC-MS analysis.

Component	Percentage (%)
Allyl sulfide	11.12
Allyl disulfide	46.15
Diallyl trisulfide	31.52
Diallyl tetrasulphide	10.60
2-Butyne, 1,4-bis(ethylthio)	0.16
1,3-Butadiene, 3-methyl-1,1-bis(methylthio)	0.09
1-Allyl-3-(2-(allylthio) propyl) trisulfane	0.20

Optical rotation: 0.8, Refractive index: 1.4714, Specific gravity: 1.0417

Table 2 provides a detailed chemical analysis of caraway essential oil conducted using gas chromatography-mass spectrometry (GC-MS). The major constituents of the oil are clearly identified, along with their respective percentage compositions. The key components include α-pinene (0.31%), sabinene (0.21%), β-myrcene (0.56%), 3-carene (0.09%), limonene (44.34%), linalool (0.22%), α-terpineol (0.08%), cis-dihydrocarvone (0.93%), trans-dihydrocarvone (0.18%), and d-carvone (53.10%). For some of these compounds, the table also specifies the acceptable concentration limits. For example, β-myrcene should be present within the range of 0.1 to 1.0%, limonene between 30.0 to 45.0%, and trans-dihydrocarvone should be less than 2.5%.

Table 2: Black caraway essential oil composition by GC-MS analysis.

Composition	Percentage (%)	Acceptable Limit (%)
α-Pinene	0.31	-
Sabinene	0.21	-
β-Myrcene	0.56	0.1 - 1.0%
3-Carene	0.09	-
Limonene	44.34	30.0 - 45.0%
Linalool	0.22	-
α-Terpineol	0.08	-
Cis-Dihydrocarvone	0.93	-
Trans-Dihydrocarvone	0.18	Less than 2.5%
d-Carvone	53.10	50.0 - 65.0%

Optical Rotation: 0.74, Refractive Index: 1.485, Specific Gravity: 0.910

The GC-MS analysis of Black Seed Essential Oil, as shown in Table 3, reveals its complex chemical composition. The major constituents of the oil include Trans-Anethole (14.4%), Thymoquinone (10.9%), p-Cymene (8.3%), and Carvacrol (7.4%). Additionally, the analysis identified significant amounts of α-Thujene (5.4%), Longifolene (4.5%), Limonene (3.2%), Sabinene (2.1%), and Estragole (2.1%).

Table 3: Black seed essential oil composition by GC-MS analysis.

Composition	Percentage (%)
Myrcene	0.9
b-Pinene	1.2
3-Methyl Nonane	0.8
2(1H)-Naphthalenone	1.5
Sabinene	2.1
a-Pinene	1.8
Thymol	6.4
Limonene	3.2
1-Ethyl-2,3-dimethyl benzene	0.4
a-Phellandrene	1.9
n-Hexadecane	0.4
borneol	0.9
n-Decane	0.7
1,8-Cyneole	1.2
1-Methyl-3-propyl benzene	0.5
Carvone	1.9
Uvidine	2.4
Terpinyl acetat	0.09
Terpinolene	0.2
Fenchone	0.8
Nerol	0.8
Carvacrol	7.4
p-Cymene-8-ol	0.6
Apiole	1.7
Estragole	2.1
Dihydrocarvone	0.8
p-Mentha-2,8-dien	0.9
longicyclene	1.3
a-Longipinene	0.9
α- Thujene	5.4
Trans-Anethole	14.4
g-Terpinene	0.7
Myristicin	1.8
camphor	1.3
n-Tetradane	0.3
Longifolene	4.5
p-Cymene	8.3
β-Cyclocitral	2.1
Thymoquinone	10.9
Terpinen-4-ol	1.3
Anisaldehyde	2.4

Optical Rotation: 0.74, Refractive Index: 1.485, Specific Gravity: 0.910

The oil also contains a variety of other compounds, albeit in lower percentages. These include Myrcene (0.9%), b-Pinene (1.2%), 3-Methyl Nonane (0.8%), 2(1H)-Naphthalenone (1.5%), a-Pinene (1.8%), Thymol (6.4%), 1-Ethyl-2,3-dimethyl benzene (0.4%), a-Phellandrene (1.9%), n-Hexadecane (0.4%), borneol (0.9%), n-Decane (0.7%), 1,8-Cineole (1.2%), 1-Methyl-3-propyl benzene (0.5%), Carvone (1.9%), Uvidine (2.4%), Terpinyl acetate (0.09%), Terpinolene (0.2%), Fenchone (0.8%), Nerol (0.8%), p-Cymene-8-ol (0.6%), Apiole (1.7%), Dihydrocarvone (0.8%), p-Mentha-2,8-dien (0.9%), longicyclene (1.3%), a-Longipinene (0.9%), g-Terpinene (0.7%), Myristicin (1.8%), camphor (1.3%), n-Tetradane (0.3%), Terpinen-4-ol (1.3%), and Anisaldehyde (2.4%).

Result

Histological analysis of gill, kidney, and intestine tissues

Microscopic examination of gill tissues

In the microscopic examination of the gills, the primary lamellae with a cartilaginous structure along with the secondary lamellae were observed in the control group and the garlic group, and no specific pathological lesions were observed in the gill tissue. In the black seed, black caraway, and the combination groups, the gill tissue showed mild hyperplasia of the epithelial cells of the secondary lamellae, deformation of the club-shaped secondary lamellae, and mild infiltration of mononuclear inflammatory cells, particularly lymphocytes and plasma cells, as well as mild edema in the secondary lamellae (Figs. 1 to 5).

Microscopic examination of kidney tissue

In the microscopic examination of the kidney tissue in the control group and the black seed group, the normal structure of the tubules, glomeruli, and the interstitial tissue was observed.

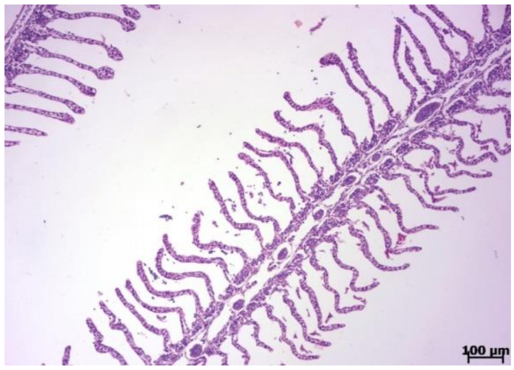

Figure 1: Gill tissue in the control group, HandE staining.

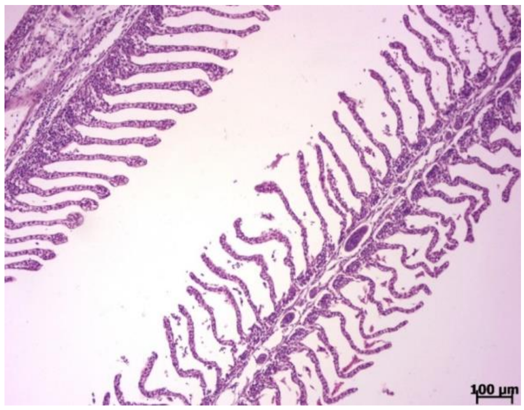

Figure 2: Gill tissue in the garlic group, HandE staining.

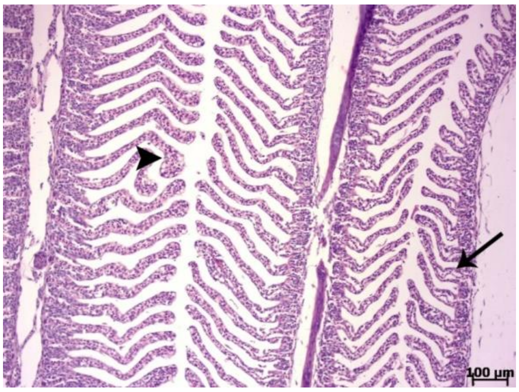

Figure 3: Club-shaped deformation of secondary lamellae (arrow) and mild edema (arrowhead) in the secondary lamellae in the black seed group, HandE staining.

Figure 4: Mild increase in epithelial cells of secondary lamellae and their attachment (arrowhead) along with mild edema (arrow) in secondary lamellae in the black seed group, HandE staining.

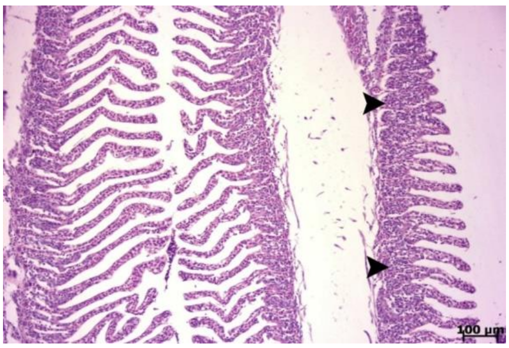

Figure 5: Mild infiltration of mononuclear inflammatory cells (arrowhead) in the combination group, HandE staining.

In the black caraway and the combination groups, the kidney tissue was almost normal, and only very mild proteinuria was observed in the form of eosinophilic hyaline casts within the lumen of the renal tubules. In the combination group, in addition to moderate proteinuria, mild and focal hemorrhages were also observed in some sections of the kidney tissue (Figs. 6 to 11).

Microscopic examination of intestinal tissue

The microscopic examination did not show any pathological changes in the intestinal tissue in any of the groups. In the cross-section of the intestine, the villi appeared as finger-like projections of the intestinal mucosa protruding into the lumen.

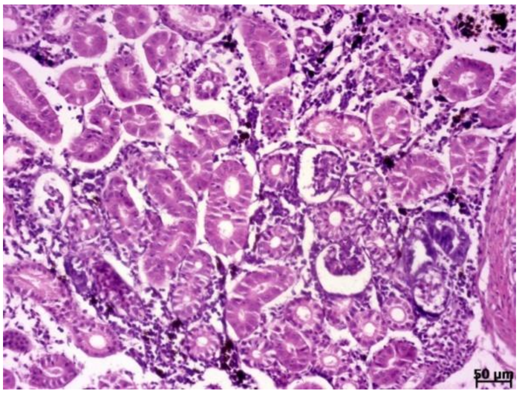

Figure 6: Normal kidney tissue in the control group, HandE staining.

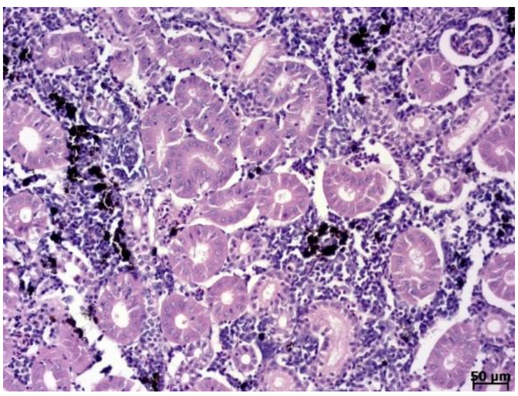

Figure 7: Normal kidney tissue in the cumin group, HandE staining.

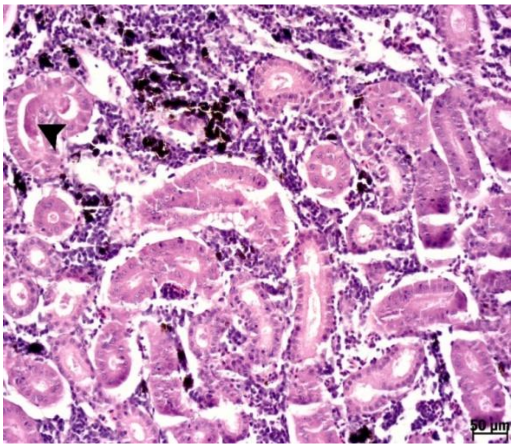

Figure 8: Minimal proteinuria observed as eosinophilic hyaline casts in the lumen of renal tubules (arrow) in the kidney tissue of the black seed group, HandE staining.

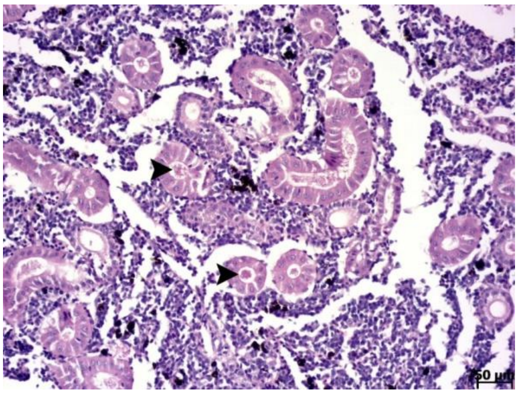

Figure 9: Minimal proteinuria observed as eosinophilic hyaline casts in the lumen of renal tubules (arrows) in the kidney tissue of the black seed group, HandE staining.

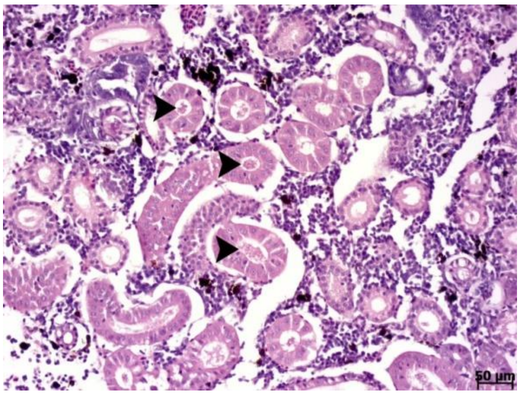

Figure 10: Moderate proteinuria is observed in the form of eosinophilic hyaline casts in the lumen of the urinary tubules (arrows) in the kidney tissue of the combination group, HandE staining.

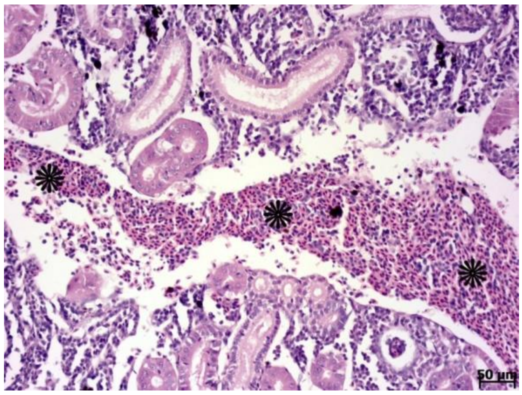

Figure 11: Hemorrhage (*) in the kidney tissue of the combination group, HandE staining.

These villi were covered with a simple columnar epithelium, primarily composed of enterocytes and a number of goblet cells. In the black seed and to some extent the black caraway groups, the length of the villi was observed to be longer compared to the control group and the other groups. In the combination group, in addition to the increased length of the villi compared to all other groups and their overlapping, the hyperplasia of the goblet cells was also evident (Figs. 12 to 16).

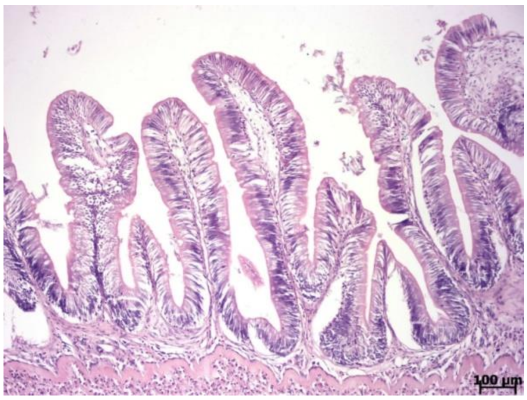

Figure 12: Normal intestinal tissue in the control group, HandE staining.

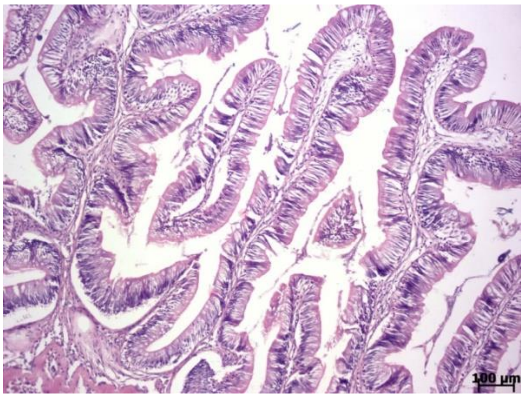

Figure 13: Increased villus length in the intestinal tissue of the cumin group, HandE staining.

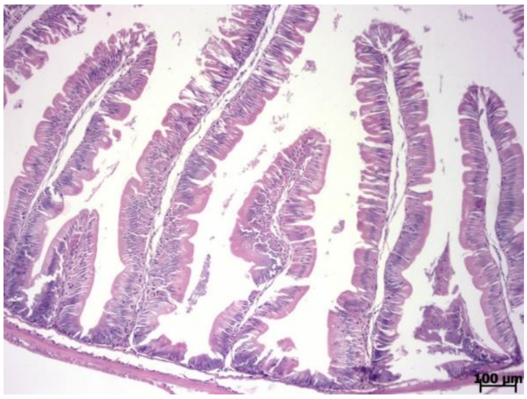

Figure 14: Intestinal tissue in the black seed group, HandE staining.

Overall, the microscopic examination of the gill tissue in the different groups showed that except for very mild pathological changes, including mild epithelial cell hyperplasia, clubbing of the secondary lamellae, mild infiltration of mononuclear inflammatory cells, and mild edema in the secondary lamellae, no other lesions were observed, and these mild lesions were negligible. In the combination group, in the intestinal tissue, the increase in the length of the villi and the increase in the number of enterocytes in the villus epithelium were very significant. However, a mild to moderate degree of proteinuria in the form of hyaline casts in the urinary lumen was observed in this group, indicating the onset of mild damage to the kidney tissue.

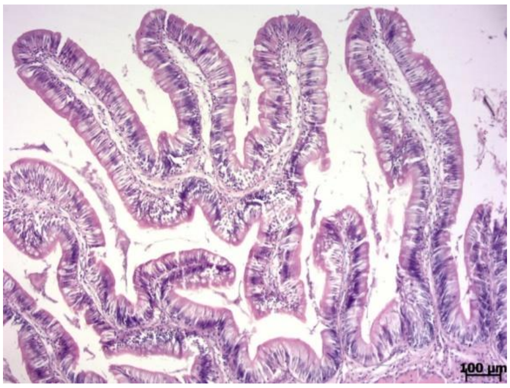

Figure 15: Intestinal tissue in the black seed group, HandE staining.

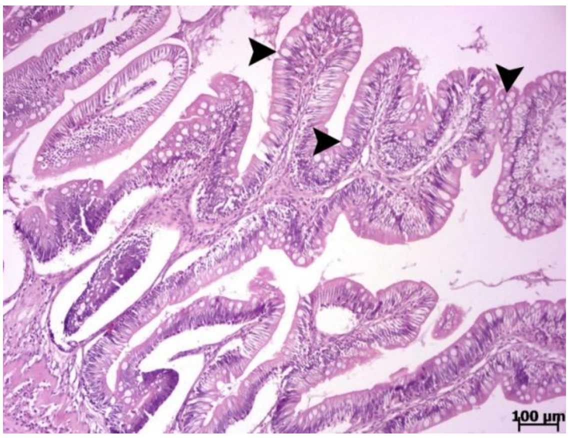

Figure 16: Increased villus length and overlap, hyperplasia of mucosal cells (arrows) in the combination group, HandE staining.

Discussion

Histological examinations play an important role in revealing the improvement of tissues and fish cells under adverse conditions (Ling *et al*., 2019). Antioxidants, anti-inflammatories, and essential oils derived from plants are used as feed additives in fish diets due to their beneficial properties (Abdel-Latif *et al*., 2020; Dawood *et al*., 2020). Plant-based supplements can have a positive impact on gut health. However, the effects of these supplements depend on the type of compound, dose, and duration of consumption. Furthermore, different compounds may interact with each other and produce varying effects (Foysal *et al*., 2019; Mohammad, 2020). Based on the research findings, no pathological changes were observed in the intestinal tissue of any group. The intestinal villi were finger-like and covered with a simple columnar epithelium. In the garlic and black seed groups, the villi were longer compared to the control group. In the combined group, the villi were longer, and there was hyperplasia of the mucosal cells. Overall, only minor and negligible changes, such as mild hyperplasia and inflammation, were observed in the gill tissue. In the combined group, the intestinal villi were significantly longer, and the number of enterocytes was higher, which is noteworthy. This group also showed mild to moderate proteinuria, indicating initial kidney damage. The study by Agbebi *et al*. (2013) showed that a diet containing 30% garlic did not cause any observable lesions in the liver, intestine, or gills of African catfish. Similarly, Öz *et al*. (2024) reported that garlic oil had a protective effect against cypermethrin toxicity in Nile tilapia, as the garlic oil groups showed fewer histological lesions in the gill, liver, brain, and muscle tissues compared to the cypermethrin group. The findings of the present study are consistent with these previous results and demonstrate longer intestinal villi in the garlic group compared to the control and other groups.

Multiple other studies have shown that supplementation with essential oils of ginger, bay leaf, thyme, coriander, and savory were able to improve intestinal histomorphology without negative effects on liver tissue (Chung *et al*., 2021; Mazlum *et al*., 2022; Lakwani *et al*., 2022; Özel *et al*., 2022; Das *et al*., 2023; Dokou *et al*., 2023). Chung *et al*. (2021) found that adding 0.5 ml/kg of ginger essential oil to the diet of Nile tilapia improved intestinal histomorphology without negatively affecting liver histomorphology. The study by Mazlum *et al*. (2022) showed that adding 0.3% bay leaf (Laurus nobilis) essential oil to the diet of tilapia improved liver and intestinal tissues without causing any pathological changes. The researchers recommend the use of 0.3% bay leaf essential oil as a feed additive in tilapia aquaculture. Lakwani *et al*. (2022) found no histological changes in the organs of fish fed the dietary supplement, but the supplement increased the proinflammatory cytokine IL-1β in the intestine, indicating enhanced intestinal immunity. They recommend a supplementation level of 1-2% for 30 days. Özel *et al*. (2022) reported that supplementing the diet of European sea bass with thyme essential oil positively affected intestinal histomorphology without negatively impacting growth. In a study by Das *et al*. (2023) on Nile tilapia, coriander oil supplementation reduced the vacuolar structure of the liver, indicating a positive effect on liver histomorphology. Additionally, the intestinal villi were longer in the coriander oil supplemented groups compared to the control, indicating improved intestinal histomorphology. Similarly, in a study by Dokou *et al*. (2023) on rainbow trout, savory oil supplementation resulted in longer intestinal villi and more goblet cells in the anterior intestine compared to the control group. Based on these collective findings, the use of selected herbal feed additives such as garlic, black seed, ginger, bay leaf, thyme, coriander, and savory should be further investigated as potential dietary supplements in aquaculture. Given the mild to negligible histological changes observed, these natural products appear to be safe and tolerable alternatives to conventional additives.

Conclusion

The histological examination further confirmed the benefits of garlic essential oil, as the garlic group showed the least changes in the gill, kidney, and intestinal tissues of rainbow trout. In contrast, the combined group showed the most adverse effects on the fish tissues. Consequently, the findings of this study indicate that dietary supplementation with encapsulated garlic essential oil resulted in the healthiest tissue in rainbow trout among the three essential oils evaluated. These plant compounds warrant further investigation for their potential applications in sustainable aquaculture practices. In conclusion, the study's findings demonstrate that dietary supplementation with encapsulated garlic essential oil had the most beneficial effects on the histological parameters of rainbow trout tissues compared to the other essential oils tested. The garlic essential oil group exhibited the least changes in the gill, kidney, and intestinal tissues, while the combined group showed the most adverse effects. These results suggest that garlic essential oil has promise as a natural additive for improving fish health and welfare in aquaculture systems. Further research is needed to fully explore the potential applications of these plant-derived compounds in sustainable aquaculture practices.

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