AQUATIC RESEARCH

AQUATIC RESEARCH
E-ISSN 2618-6365

Aquat Res 6(3), 166-174 (2023) • https://doi.org/10.3153/AR23017 *Research Article*

Effects of black cumin and grape powder mix on growth performance and hepatopancreas histology in third instars of *Pontastacus leptodactylus*

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Cite this article as:

Mazlum, Y. Yazıcı, M., Ürkü, Ç., Bahadır Koca, S. (2023). Effects of black cumin grape powder mix on growth performance and hepatopancreas histology in third instars of *Pontastacus leptodactylus*. *Aquatic Research*, 6(3), 166-174. https://doi.org/10.3153/AR23017

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Submitted: 11.04.2023

Revision requested: 18.05.2023

Last revision received: 20.05.2023

Accepted: 21.05.2023

Published online: 19.06.2023

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Available online at
http://aquatres.scientificwebjournals.com

ABSTRACT

This study investigated the effects of dietary supplementation of combined black cumin (BC) and grape seed (GS) powder at different rates (0%, 1%, 2%, and 4%, respectively) on growth performance and hepatopancreas histology of third instar Turkish narrow-clawed crayfish (*Pontastacus leptodactylus)*. A total of 180 third instar of crayfish with an initial mean weight of (0.06 g ±0.01 g) and mean length (11.0 mm ±0.01) were randomly assigned to four treatment groups with three replications in each group and at a density of 15 crayfish per replicate. Crayfish were fed an experimental diet rate of at 10% of total body weight for 84 days. Growth performance parameters were improved significantly with BC and GS powder supplementation ($P<0.05$), while survival rates did not differ among all groups ($P>0.05$). Weight gain, specific growth rate and feed conversion rate of the 1% group tended to be better than the other groups, with significant differences among groups ($P<0.05$). The number and volume of B cells increased significantly with the increase in the amount of (BC and GS) added to the feed (1, 2, and 4%) in the hepatopancreas. In addition, it was determined that the R cells, which absorb nutrients, are dense in the hepatopancreas tissue of the crayfish fed with 1% (BC and GS) added feed compared to the other groups. The results showed that dietary supplementation of 1% BC and GS could benefit third-instar crayfish's growth and histological data. Therefore, combining BS and GS can be recommended as a promising new feed additive for narrow-clawed crayfish culture.

Keywords: Medical plants, Freshwater Crayfish, *Nigella sativa*, *Vitis vinifera*, Growth, Survival

Introduction

The Turkish narrow-clawed crayfish (*Pontatastacus leptodactylus*) is a decapod crustacean (Decapoda: Astacidae) native to the freshwater habitat of the northeastern part of Türkiye (Crandall and Grave, 2017). *P. leptodactylus* exhibit several physical, biological, and commercial characteristics that make them suitable candidates for aquaculture (Mazlum and Yılmaz, 2006). *P. leptodactylus* play critical ecological roles as planktonic grasslands, epibenthic scavengers or prey species. They are important as indicator species for freshwater lakes, dams, and stream habitat quality (Reynolds et al., 2013) conservation biology. The fact that *P. leptodactylus* is the only domestic crayfish species in Türkiye has high economic value and is an export product increases the demand for crayfish. Türkiye has a wide distribution of water resources, and its production is based on hunting. Until now, there is no record of its cultivation in Türkiye. However, in 2015, a crayfish hatchery was built in Egirdir Institute, the first and only closed recirculating system. *P. leptodactylus* production from fishing reached 1011 tonnes in 2021 (TUIK, 2021).

The significant constraints on the cultivation of crayfish are that they have a prolonged egg incubation and larval feeding period and are susceptible to crayfish plague (Svoboda et al., 2014; Mazlum et al., 2021). *P. leptodactylus,* like many other farmed aquatic species, are more sensitive to physical and physiological conditions during their larval stages and more vulnerable to diseases (Mazlum et al., 2017). These situations are one of the most important parts of crayfish production. Success in this period directly affects crayfish production.

One of the most important factors in overcoming these challenges is proper nutrition, which is effective in maintaining the growth and health of aquatic organisms (Sönmez et al., 2020). Prepared rations provide essential nutrients for the physiological functions of aquatic organisms but also mediate the intake of other components that may positively affect health (Li and Gatlin, 2004). Today, studies are carried out in which many functional feed additives, including probiotics (Hoseinifar et al., 2019), prebiotics (Yazici et al., 2020), medicinal and aromatic plants (Yazici et al., 2022), macro/microalgae etc. (Hoseinifar et al., 2022) are tested in order to increase the growth performance and survival rate in aquaculture.

Medicinal plants and their derivatives are considered promising as feed additives in aquaculture due to their many properties (Li et al., 2019). The most prominent features of medicinal plants are that they have bioactive compounds with no side effects, inexpensive, biodegradable, easy to access, and environmentally friendly (Tadese et al., 2022). For this reason, medicinal plants have been widely adapted for a long time in aquaculture as they improve the performance and health status of aquatic animals (Hoseinifar et al., 2018; Jeyavani et al., 2022; Mariappan et al., 2023).

More than 60 different medicinal plants are considered feed additives in aquaculture (Bulfon et al., 2015). One of these plants, Black cumin (*Nigella sativa*), is a medicinal plant native to Southern Europe, North Africa, and Southwest Asia. This plant is grown in many world countries, such as Iran, Pakistan, India, Türkiye and Saudi Arabia, the Middle East Mediterranean region, and Southern Europe (Yılmaz et al., 2022). *N. sativa* powder is a rich source of protein, crude oil, crude fibre and macro minerals and is composed of protein (26.7%), lipid (28.5%), carbohydrates (24.9%), and fibre (8.4%), and ash (4.8%). The rest comprises sugar mineral substances (Nickavar et al., 2003). The main active ingredients are thymol, thymohydroquinone, thymoquinone, carvacrol and thymoquinone (Abd El-Hack et al., 2021). It was exhibited that black cumin and its derivatives have some advantageous features such as immunostimulant (Dorucu et al., 2009; Altunoglu et al., 2017), growth-promoting (Öz et al., 2018; Bektaş et al., 2019), antioxidants (Yonar, 2017), antimicrobial activities (Khondoker et al., 2016; Hal et al., 2021) for farmed fish. Additionally, using *N. sativa* or its derivatives as a supplement in fish diets protects against fish pathogens (Khondoker et al., 2016; Hal et al., 2021).

Another medicinal plant is grape seed, which is rich in polyphenolic compounds with potent biological effects. These polyphenols, consisting of flavonoids and phenolic acids (Mekrinakhi et al., 2020), have antioxidant (Souza et al., 2019) and antibacterial activity (Ahmad et al., 2014). Grape seed has a complex composition, and the constituents of the composition were determined as 11% protein, 16% fat, 40% fibre, and 7% phenolic compounds. Some researchers have reported that grape seed extract has a positive effect on growth performance (Kesbiç and Yiğit, 2019), antioxidant defence (Mousavi et al., 2020) and immune system in farmed fish (Arslan et al., 2018; Mehrinakhi et al., 2020).

It is thought that medicinal plants as feed additives will contribute to this growth in yield, quality, and income increase. Furthermore, some studies have reported that combining herbal powders is more effective than a single herb administration (AbdelWahab and El-Bahr, 2012). However, there is limited research on combining herbal extracts as a new strategy to enhance crayfish performance and improve health status. Hence, the present study investigated the synergistic ef-

fect of dietary administration of *N. sativa* and *V. vinifera* mixture on the growth performance and some hepatopancreatic histological parameters of narrow-clawed crayfish *P. leptodactylus*.

Materials and Methods

Ovigerous female (n=45) narrow-clawed crayfish weighing 41.8 g and a total length of 109.6 mm were collected from Lake Eğirdir in 2023, using fyke-net (16 mm mesh size) fed with frozen fish. The caught female crayfish with eggs were packed and shipped by bus to Iskenderun Technical University Hatay Fisheries Research Facilities. Egg-laying female crayfish were stocked in three PVC tanks containing 15 crayfish to acclimate to the experimental environment before starting the research. Potassium permanganate was applied every 3 days to prevent fungal infection. During adaptation, crayfish were fed with sea bass commercial feed. Egg hatching was done in the rearing unit of Iskenderun Technical University, Faculty of Marine Sciences and Technology.

Length (mm) and weight (g) were measured by subsampling from healthy hatched crayfish (n=50) and placed in 12 tanks. A sizing board was used to measure the total length (TL, mm) of the crayfish, and a digital scale with an accuracy of (0.01 g) was used to measure their wet weight (WW, g). One hundred and eighty (n=180) healthy third instar crayfish chosen for the experiment (mean weight 0.06 g ±0.01 g and length 11 mm ±0.01) were randomly placed in four treatment groups of 0% (control group), 1%, 2%, and 4%, respectively. Experimental groups were performed in triplicate with 15 crayfish in each tank. The experimental period was 84 days. Small pipes and pieces of mesh are placed at the bottom of the tank to reduce the contact of the crayfish with each other.

Black cumin (*Nigella sativa*) (BC) and grape seed (GS) powder were purchased from herbalists in the Iskenderun district of Hatay province. The diet in the powder was added to an experimental commercial sea bass diet (0.5 g BC+0.5 g GS powder; 1:1) at a 1g/100 g feed rate. The control group's diet did not include any supplements. The mixture was turned into Alphie1 pellet feed with the 3D mixing feature. The prepared feeds were stored in plastic containers at +4°C. In the experiment, crayfish in all groups were fed ad libitum daily for 84 days. The amount of feed was 10% of the total live weight. Inedible feeds and crayfish droppings were siphoned away from all tanks not to deteriorate the water quality.

During the experiment, temperature (ºC) and the dissolved oxygen (mg/L) in the water were measured every morning (08:00) using an oxygen meter. pH values are measured weekly with a pH meter. The water used in the experiment was carried out as a continuously flowing aquaculture system.

The experiment's measurement of growth performance parameters was not done for the first 30 days in order not to harm the offspring due to their very small size, and measurements were made on the 60th and 84th days. At the end of the experiment, crayfish for all groups were counted and weighed to determine the weight, weight gain, specific growth rate, survival rate, and feed conversion by the following equations.

Weight Gain (WG, g)

= Final crayfish weight (g) – Initial crayfish weight (g)

Weight gain rate (WGR, %)

$$= \frac{\text{Final crayfish weight (g)} - \text{Initial crayfish weight (g)}}{\text{Initial crayfish weight (g)}} * 100$$

Specific Growth Rate (SGR)

$$= \frac{\text{In Final weight (g)} - \text{In Initial weight (g)}}{\text{days}} * 100$$

Feed conversion ratio (FCR)

$$= \frac{\text{Total feed consumption (g)}}{\text{Weight gain (g)}}$$

Survival Rate (SR, %)

$$= \frac{\text{Number of crayfish at the end of experiment}}{\text{Number of crayfish at the beginning of experiemnt}} * 100$$

On the final day of the experiment, hepatopancreas samples (n= 3) were immediately taken from each treatment group to perform the histological analysis. The samples were fixed in 10% phosphate-buffered formaldehyde, dehydrated in graded serial ethanol, and embedded with paraffin. Subsequently, 4-µm-thick tissue sections were made using a rotary microtome, stained with hematoxylin and eosin (H&E) and examined under a light microscope (Nikon E 600) using a digital camera (Ettore et al., 2017).

The parameters examined in the experiment were tested for significance at the 5% level using a one-way analysis of variance (ANOVA) ($P<0.05$). Results are given as mean ± standard deviation of the mean (SD). In the data analysis, the homogeneity of variance (ANOVA) test was applied to the data

before comparisons were made among the treatment groups. Data results were tested with the Shapiro-Wilk test for normal distribution. Statistical analyses of the data obtained in the experiment were done using the SPSS 17.0 statistical package program (SPSS INC. Chicago, IL, USA).

Results and Discussion

The water quality values used in this study were within acceptable ranges for crayfish growth (Mazlum, 2007). No statistical difference was observed in mean water temperature, dissolved oxygen and pH (P>0.05) during the experiment. The water temperature used in the experiment was average (20.7-21.30 °C), and the dissolved oxygen level varied between 5.8-7.6 mg/l depending on the water temperature during the 84-day experiment period. Rapid aquaculture growth is one factor that significantly affects both feed and animal welfare. In aquaculture, many stress factors, including bacterial infections, have been shown to alter the feeding and growth of aquatic organisms (Farag et al., 2021). Accordingly, in this study, we evaluated whether black cumin seed and grape seed powder affected the growth, survival, and histology of hepatopancreas in narrow-clawed crayfish.

Medicinal plants, which have long been used to improve human nutrition, have recently received significant attention in livestock, poultry, and aquatic animals (El-Deep et al., 2019; Saleh et al., 2019; Shekarabi et al., 2020). Medicinal plants have also attracted the attention of aquaculture researchers every day due to their beneficial effects on fish immunity, antistress, antioxidant, growth promoter and feed digestibility (Bulfon et al., 2015; Hal et al., 2021; Yılmaz et al., 2022). Such additives can increase the growth of beneficial microbial colonies in the digestive tract, thereby improving growth parameters by increasing feed intake and weight gain of aquatic species (Jeyavani et al., 2022; Mariappan et al., 2023). In addition, increased resistance of aquatic species to infectious diseases following the application of different medicinal plants has also been previously demonstrated (Ahmad et al., 2013; Khondoker et al., 2016; Mehrinakhi et al., 2020).

When the study results were examined, it was shown that BC and GS powder did not negatively affect crayfish's growth performance when 1%, 2% and 4% BC and GS were added to the diet. In contrast, adding different levels of BC and GS powder to the diet significantly affected weight gain, specific growth rate, feed conversion rate and productivity of third-stage crayfish, but not survival rate (Table 1). However, the BC and GS ratio increase partially decreased the feed conversion ratio and survival rate. These results agreed with those obtained by Niroomand et al. 2020, Öz et al., 2018; Bektaş et al., 2019; Latif et al., 2020; Youssif et al., 2020 who found that supplementation of BC and GS powder in fish feed. Since studies on crustaceans are limited, comparisons with other species have been made. This study determined that the experimental feeds prepared to contain different ratios of BC and GS positively affected the growth performance of the experimental group *P. leptodactylus* third instar. While the values in the 1% group were found to be significant compared to the changes in the weight gains of the other groups (P<0.05), the change in the weight gain (%) values was not significant (P>0.05) (Table 1).

Table 1. Growth performance and feed conversion ratio in narrow clawed crayfish diets for 84 days.

Parameters	Control	BC+GS 1%	BC+GS 2%	BC+GS 4 %
Initial weight (IW, g)	0.06 ±0.01	0.06 ±0.01	0.06 ±0.01	0.06 ±0.01
Final weight (FW, g)	0.47 ±0.14^{a}	0.60 ±0.09^{b}	0.40 ±0.09^{a}	0.40 ±0.11^{a}
Weight gain (WG, g)	0.41 ±0.14	0.54 ±0.09	0.34 ±0.09	0.34 ±0.11
Weight gain (WG, %)	683.33	900.00	566.66	700.00
Initial length (ITL, mm)	11.00 ±0.01	11.00 ±0.01	11.00 ±0.01	11.00 ±0.01
Final length (FTL, mm)	27.67 ±3.20^{a}	28.00 ±1.56^{a}	26.00 ±1.56^{b}	25.08 ±1.78^{b}
Length increment (LC, mm)	16.67 ±3.20^{a}	17.00 ±1.56^{a}	15.00 ±1.56^{b}	14.08 ±1.17^{b}
Feed conversion rate (FCR)	1.17 ±0.01^{a}	0.96 ±0.02^{b}	1.34 ±0.02^{c}	1.20 ±0.01^{a}
Specific growth rate (SGR, %)	0.024 ±0.000^{a}	0.027 ±0.0001^{b}	0.022 ±0.0001^{a}	0.022 ±0.0001^{b}
Survival rate (SR, %)	44.00	75.55	66.66	53.33

IW: initial weight (g), FW: final weight (g), ITL: initial length (mm), FTL: final length (mm), WG: percent weight gain (%), SGR: specific growth rate (%), FCR: feed conversion ratio, SR: survival rate (%). BC and GS

Similar to the results of the current study, *Oreochromis niloticus* fed a 2% black seed supplement diet showed an improved growth rate (Diab et al., 2008). An increase in growth performance was reported in *Cyprinus carpio* larvae fed with a 1% black seed ration (Al-Dubakel, 2012). Similarly, growth rates were increased in *Oncorhynchus mykiss* larvae fed with diets containing 1% and 1.3% black seed oil been reported (Öz et al., 2018). Previous studies have shown that weight gain and growth rate are directly related to the animal's capacity to digest and absorb nutrients (Magouz et al., 2020; Mazlum et al., 2021). The improvement in the growth performance of BC and GS has been attributed to its various bioactive substances (Nickavar et al., 2003; Khondoker et al., 2016; Arslan et al., 2018).

Many environmental and biological factors influence water quality in crayfish farming (Eversole et al., 2004; Roessink et al., 2017). Growth and survival are closely related to water quality. Important water quality variables are dissolved oxygen, pH, hardness, nitrite, and ammonia. Of these, dissolved oxygen is the most important, and low oxygen may be directly or indirectly responsible for the death of crayfish more than any other factor (Foysal et al., 2020). In addition, considering all these features, it is known that temperature significantly affects oxygen levels. In our study, it was observed that the water quality parameters were within acceptable limits. Moreover, feed quality and feed components are important parameters in the growth of crayfish (Halver and Hardy, 2003). This study observed that the water quality did not change with the addition of medical feed additives to the feed of experimental crayfish.

Crayfish store large amounts of lipids (energy) in the hepatopancreas for oocyte development, survival, digestion, nutrient absorption, immunity, and metabolism (Lu et al., 2023). Several studies have shown that the hepatopancreas undergoes structural changes due to feed additives (Jahromi et al., 2021). Therefore, the general nutritional status of crustaceans can be evaluated by histological studies in the hepatopancreas (Jahromi et al., 2021; Lu et al., 2023). Previous studies have shown that hepatopancreatic R (resorptive) cells (R-cells) can be used to monitor the nutritional value of diets (Vogt et al., 1986; Genç et al., 2007). In this study, BS and GS powder containing different ratios showed healthy histological structures in the control and treatment groups (Figure 1) and are important for lipoprotein metabolism. Absorbent R-cells playing a role were typically found to be normal. The 1% BC and GS group was observed to provide the best recovery in the hepatopancreas (Figure 1). It was determined that the applied doses did not harm the hepatopancreatic tissue; on the contrary, there were improvements.

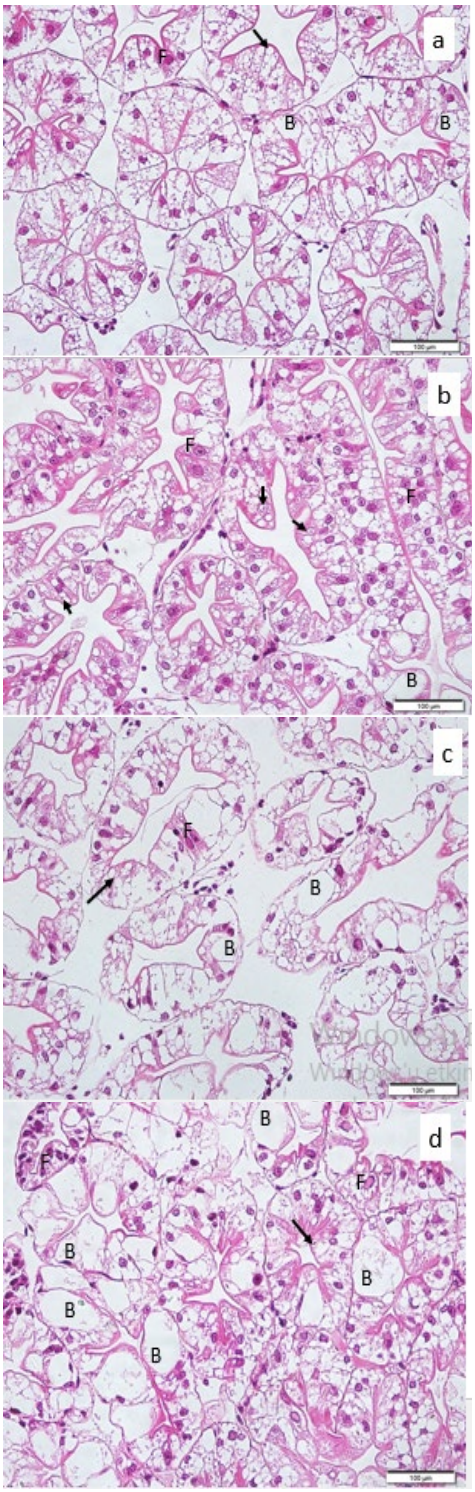

Figure 1. Light photomicrograph of sections hepatopancreatic tissues of *Pontastacus leptodactylus* crayfish fed with BC+GS supplemented feed (a): Control group, (b) 1% BC+GS, (c) 2% BC+GS, (d) 4% BC+GS (B: Blasenzellen cell, F- Fibrillenzellen or Fibrous cells, Restzellen cells-indicated by arrow) (bar: 120 µm, H&E).

Conclusion

The results indicated that BC and GS powder supplementation helps significantly increase growth performance and survival rates of *P. leptodactylus* when added to their diet. The 1% group's weight gain, specific growth rate and feed conversion rate tended to be better than the other groups, with significant differences. The use of BC and GS powder together is suggested as a potential candidate for use as a feed additive in intensive aquaculture applications to prevent stress-related losses and ultimately increase production.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: Animal care and experiments were carried out considering national and international guidelines.

Funding disclosure: -

Acknowledgments: -

Disclosure: -

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