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AQUAPONIC CORNER

Control of ectoparasitosis in carp (*Cyprinus carpio*) induced by *Gyrodactylus elegans* (Monogenea) with garlic (*Allium sativum*) and onion (*Allium cepa*) extracts

Hijran Yavuzcan Yildiz, Suleyman Bekcan

Department of Fisheries and Aquaculture, Ankara University, Ankara, Turkey

E-mail: yavuzcan@ankara.edu.tr

Abstract – One of the constraints in fish disease management in aquaponic systems is related to undesired effects of chemicals on fish, plants and beneficial bacteria. Plant-derived compounds with nontoxic features to fish, plants, and microflora provide an alternative treatment strategy against the harmful pathogens in the aquaponic system. The present study assessed the antiparasitic activity of garlic (*Allium sativum*) and onion (*A. cepa*) extracts against *Gyrodactylus elegans* (Monogenea) *in vivo* and *in vitro*, and physiological stress responses in carp, *Cyprinus carpio*, treated with these extracts in an aquaponics system. Garlic and onion extracts exhibited *in vitro* antiparasitic activity against *G. elegans*. The mean survival time of *G. elegans in vitro* ranged from 30 sec to 6 min depending on the concentration and exposure time both for garlic and onion extracts. For garlic extract EC₅₀ (median effective concentration) was 8.37±4.75 mg/mL in 3 min exposure and for onion extract 4.72±7.10 mg/mL. These concentrations were *in vivo* tested in carp heavily infected with *G. elegans* as a single application for 3 min. *In vivo* treatment of carp with garlic and onion extracts reduced *G. elegans* found on the skin by 14.4% and 19.8%, respectively. In both treatment groups, the physiological stress response of carp was mild based on the alterations in the secondary stress indicators (hematocrit, plasma glucose, and lactate). The stress indicators of carp returned to normal levels after an hour recovery in freshwater. The antiparasitic potential of onion and garlic extracts may be considered as an alternative treatment to reduce Monogenean infections in aquaponic systems.

Keywords – aquaponics, ectoparasite, *Gyrodactylus elegans*, garlic, onion

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INTRODUCTION

Aquaponic systems integrate recirculating aquaculture (RAS) with hydroponics. The complexity of aquaponic systems entails the consideration of sustainability, including economical, technical and social aspects (König et al. 2016). The management of fish diseases is one of the challenges in aquaponic systems. Parasites particularly create serious health risks for fish and limit productivity in aquaponic systems (Goddek et al. 2015). Monogenean parasites display a single-host life cycle (Buchmann and Lindenstrøm 2012; Hutson et al. 2018) and have the ability to infect the skin and gills of marine and freshwater fish. Species of the genus

Gyrodactylus generally attach to the skin of freshwater fishes. *Gyrodactylus* spp. are characterized by being viviparous with high reproductive capacity (Stoskopf 1993). The pathogenicity of *Gyrodactylus* spp. depends on several environmental factors such as water temperature, pH and nutrient concentrations (Bakke et al. 2002). Sub-optimal water quality and other stressors in aquaponics can predispose fish to an invasion of Monogenean parasites because it is usually not possible to ensure optimal production parameters simultaneously for fish and plants.

As it is difficult to eradicate Monogeneans from fish skin or gills various externally applied agents are used to control

Monogenean species. The application of agents, mainly chemicals involves some questions on environmental contamination and human health concerns (Fridman et al. 2014). The antiparasitic chemicals have an impact on the environment in terms of lethal or sub-lethal effects on non-target organisms. Treatments with chemicals may lead to accumulation in fish tissue and disruption of the natural microflora of the fish (Wunderlich et al. 2017). Specifically, in aquaponics, the parasite treatments may have harmful effects on the rest of the system, including the biofilter, nitrification biofilm, and plants, causing limitation of disease treatment (Goddek et al. 2015; Yildiz et al. 2017).

Thus, in recent years natural plant products are taken into consideration to control parasites in aquaculture to eliminate the problems due to chemical use. In aquaponics, one effective treatment option against the ectoparasite invasions is immersion or bath with plant-derived compounds due to the complexity of the system. The effects of extracts, essential oils and bioactive metabolites from diverse terrestrial and aquatic plants on fish parasites have been studied as an alternative treatment against different parasite species of fish (Wang et al. 2011; Wu et al. 2011; Lu et al. 2012; Ramudu and Dash 2013; Valladao et al. 2015; Tavares-Dias 2018). With rich bioactive compounds, garlic (*Allium sativum*) and onion (*A. cepa*) have been used for therapeutic purposes since ancient times (Anthony et al. 2005; Lanzotti 2012). Hyun Kim et al. (2018) studied the effects of garlic on *Cryptocaryon irritans* (Ciliates) found on guppies (*Poecilia reticulata*) *in vitro* and *in vivo*, reporting a failure to cure. Fridman et al. (2014) revealed that the treatment of *Gyrodactylus turnbulli* on guppy skin with garlic extract (bath at a concentration of 7.5 and 12.5 mL for 1 h) resulted in the detachment of parasite and death, indicating reduced parasite prevalence. Apart from external applications, the administration of plants or plant-derived substances in the feed was evaluated for their anti-parasitic activity. Militz et al. (2013) disclosed that garlic extract as a supplement to the diet can prevent *Neobenedenia* sp. (Monogenea) infection in farmed barramundi (*Lates calcarifer*). In line with this study, Martins et al (2002) reported that garlic powder in the diet of cultivated pacu (*Piaractus mesopotamicus*) resulted in a significant reduction of *Anacanthorus penilabiatus* (Monogenea) in the gills. On the other hand, the treatment of fish with various substances, even herbal ones may be a source of stress, impairing welfare. The welfare status of fish, as measured by the indices of stress can be unveiling (Roque et al. 2010). The secondary stress response can be expressed by the alteration in blood profile as the physiological effects of factors released in the primary response phase affect the metabolism (Kjartansson et al. 1988; Yildiz and Pulatsu 1999). Stress induces changes in energy metabolism and blood cells as reflected by the changes in baseline stress indicators including plasma glucose, lactate and hematocrit levels (Ellis et al 2012).

The present study tested the possible use of *in vitro* antiparasitic activity of garlic and onion extracts against *Gyrodactylus elegans* (Monogenean) recovered from carp (*Cyprinus carpio*) skin. *In vivo* the effects of immersion

treatments with garlic and onion extracts on parasite reduction in skin and physiological stress response of carp (*Cyprinus carpio*) were evaluated.

MATERIAL AND METHODS

Fish and parasites

Carp were obtained from the one-loop aquaponic systems producing carp and mint (*Mentha* spp.) in Ankara University, Department of Fisheries and Aquaculture. One-loop aquaponic system characteristics were explained in detail by Goddek et al. (2009). The stocking ratio of fish was 35 kg/m³ in the fiberglass fish tanks (80x60x50 cm). Water temperature was 20-22 °C, dissolved oxygen was 5.50-5.97 mg/L and pH was 6. Fish were fed with commercial rainbow trout feed (containing 45% raw protein) with 2% body weight. During routine fish health control in the aquaponics system, monogenean skin parasites on the skin were detected. The parasites were identified as *G. elegans* (Paperna 1996; Akmirza and Yardimci 2014). The scrapped mucosa samples were weighed before placement on the slides and counted for *in vitro* parasite survival tests.

Fish management and experimental protocol were approved by the ethics committee of the Ankara University with the reference number of 2019-7-72. No fish were killed while carrying out the experiments.

Extract preparation

Garlic cloves and onion were obtained from the local market, Ankara, Turkey. Garlic or onion was crushed and blended in a kitchen vortex. The blended material was weighed and 10 mg of them were diluted in 20 mL distilled water (stock solution) and vigorously mixed. This extract was freshly prepared before use and used as a stock solution.

In vitro assay

The effects of garlic and onion extract on *in vitro* parasite survival were tested. The exposure of parasite was carried out by dropping the garlic or onion extracts to the slides. Five concentrations of garlic or onion (250, 125, 100, 50 and 10 mg/mL) were tested. Different solutions from the main stock solution were prepared for each test concentration. The motility and contraction of parasites were continuously observed under a microscope and the time to death was noted. Non-motile parasites that did not respond to a touch by a needle were considered dead. The death time intervals were specified as 10-30, 30-60, 60-90 seconds and 1.5-2, 2-3, 3-4 and 4-6 minutes. Three replicates were used per concentration and each replicate contained 4 parasites. Time to death was used to determine the median effective concentration (EC50) using a Probit analysis with a maximum likelihood regression algorithm.

In vivo assays

A total of 45 carp weighing 100-150 g infested with *G. elegans* were selected from the one-loop aquaponics system for immersion treatments and physiological stress tests.

Immersion treatment

In vivo garlic and onion extract concentrations were chosen based on the minimum EC₅₀ value (EC₅₀=8.37 mg/mL for garlic and 4.72 mg/mL for onion) of garlic and onion extracts on fish. The carp used for the experiment were randomly divided into 5 treatment groups of 3 fish each and set up to different treatments as given below:

Group I: Treated with a single dose of garlic extract (EC₅₀=8.37 mg/mL) for 3 min

Group II: Treated with a single dose of garlic extract (EC₅₀=8.37 mg/mL) for 3 min followed by 1 h recovery in freshwater

Group III: Treated with a single dose of onion extract (EC₅₀=4.72 mg/mL) for 3 min

Group IV: Treated with a single dose of onion extract (EC₅₀=4.72 mg/mL) for 3 min followed by 1 h recovery in freshwater

Group V: Untreated control.

The treatments with garlic and onion extracts were performed in a 20-L plastic pot containing 5 L of solutions with extracts and three infected carp. All treatment and control groups were conducted in triplicate. After the treatments, each fish was sampled for blood analysis.

Blood analysis

Blood samples were taken as soon as exposure of carp to garlic or onion extract and subsequently after 1 h (recovery in freshwater). Control fish were handled in the same manner. Fish were slightly anesthetized in a clove oil solution (5 mg/L) for 5 min before blood sampling. Blood samples were taken by heparinized syringes from the hearth. Hematocrit was measured immediately after blood sampling. Plasma was obtained by breaking the capillary hematocrit tubes after measurements. Plasma was stored at -18 C until further analysis. Plasma glucose and lactate were measured spectrophotometrically using commercial kits (Cayman Chemicals, USA) with a Shimadzu UV-1210V spectrophotometer according to the instructions of the manufacturer.

In vivo antiparasitic efficacy assay

The anthelmintic activities of garlic and onion extracts were assessed following to blood collection. After blood sampling, skin mucus of carp in all treatments and control groups were sampled. The scrapped mucus samples (0.05 g) were placed on glass slides, and the numbers of alive parasites in the mucus were counted under a light microscope (Nikon Type 120) at 25 Å~10 magnification. The antiparasitic efficacy of each treatment and the control group were calculated according to the following formula (Wang et al. 2008):

$$\text{Antiparasitic efficacy} = \frac{A-B}{A} \times 100$$

A: The mean number of parasites in carp mucus in the treatment group

B: The mean number of parasites in carp mucus in the control group.

Statistics

Probit analysis (Finney, 1971) was used for the calculation of the EC₅₀ at the 95% confidence level (95% CL) with NCSS

statistical software. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant at $p < 0.05$.

RESULTS

In vitro parasite survival

In vitro mortality of *G. elegans* varied by both the concentrations and exposure time of garlic and onion extracts, $p < 0.05$ ($F_{2,31}=5.08$ for garlic experiment) and $F_{2,31}=20.37$ for onion experiment). EC₅₀ values of garlic and onion extracts for *G. elegans* changed by the exposure time (Table 1). For *G. elegans* garlic extract was found to be most effective with EC₅₀ of 8.37 ± 4.75 mg/mL and onion extract with EC₅₀ of 4.72 ± 7.10 mg/mL. *In vitro* cumulative mortality of *G. elegans* exposed to garlic extracts were 100% in 6 min for the concentrations tested except the concentration of 10 mg/ml (Figure 1). Cumulative mortality for onion extracts showed a similar pattern to garlic exposure (Figure 2).

Table 1. *In vitro* EC₅₀ of garlic (*A. sativum*) and onion (*A. cepa*) extracts to *Gyrodactylus elegans* (N=24 for each test concentration).

EC ₅₀	Exposure time				
	10-30 sec	30-60 sec	1-1.5 min	1.5-2 min	2-3 min
Garlic mg/mL	200.96 ±52.16	67.45 ±13.98	20.09 ±5.37	16.85 ±5.18	8.37 ±4.75
Onion mg/mL	-	6861.26 ±26.76	78.20 ±28.57	58.80 ±20.65	4.72 ±7.10

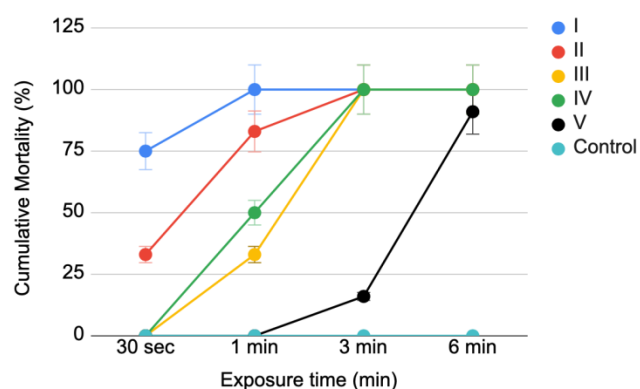


Figure 1. Cumulative mortality of *Gyrodactylus elegans* (Monogenean) exposed to garlic (*A. sativum*) extract *in vitro*. Concentrations (mg/mL) I-250; II-125; III-100; IV-50; V-10. Control: no extract exposure.

Behavioral response

The behavioral stress response in fish was apparent during the 3 min exposure of garlic at a concentration of 8.37 mg/mL or

onion at a concentration of 4.72 mg/mL extract. Carp exhibited abnormal behavior with slight tremors.

Physiological stress response

Mean values of physiological stress parameters of carp exposed to garlic extract at a concentration of 8.37 mg/mL and onion 4.72 mg/mL for 3 minutes differed from control (Table 2).

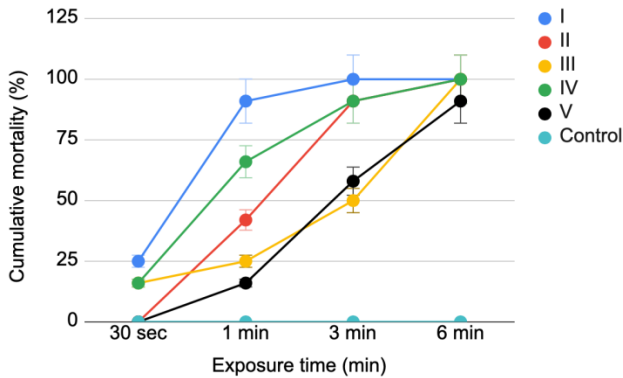


Figure 2. Cumulative mortality of *G. elegans* (Monogenean) exposed to different concentrations of onion (*A. cepa*) extract. Concentrations (mg/mL): I-250; II-125; III-100; IV-50; V-10). Control: no extract exposure.

Table 2. Stress indicators of carp (*C. carpio*) exposed to i) garlic (8.37 mg/mL) or onion (4.72 mg/mL) extract for 3 min, ii) garlic or onion extract followed by 1 h in freshwater (recovery) and iii) freshwater for 3 min (control).

Garlic / Onion Exposure	Secondary stress indicators		
	Hematocrit (%)	Plasma Glucose (mg/dL)	Plasma Lactate (mg/dL)
Garlic	40.41±2.93 ^{b*}	88.72±1.98 ^b	17.57±0.71 ^{ab}
Recovery	34.42±1.62 ^a	79.01±3.26 ^a	11.97±1.90 ^a
Onion	30.5±1.31 ^a	82.74±3.24 ^b	15.86±0.95 ^{ab}
Recovery	33.58±1.37 ^a	76.01±3.23 ^a	10.25±0.96 ^a
Control	36±2.08 ^a	78.35±3.95 ^a	9.31±0.34 ^a

*Means (N=9 fish for each group) in a column with different superscripts are significantly different (p<0.05).

Hematocrit values were significantly elevated after treatment with garlic extract (p<0.05) and declined to control levels following 1 h recovery in freshwater. Treatment with onion extract did not affect the hematocrit values (p>0.05). Plasma glucose values increased after treatment with garlic or onion extract (p<0.05) however, plasma glucose returned to control values after 1 h recovery. Plasma lactate levels of carp treated with garlic or onion extracts slightly differed from control values (p<0.05). Plasma lactate levels decreased to control levels in 1 h recovery.

In vivo antiparasitic efficacy

In vivo G. elegans found on the skin of carp were significantly decreased by garlic or onion exposure (p<0.05). Garlic and onion extracts reduced *G. elegans* on the skin 14.40% and 19.79%, respectively. The mean intensity of *G. elegans* in the mucus of carp (0.05 g) was 15.16 ± 0.56 before treatment and decreased to 12.83 ± 1.36 in garlic-treated and 12.0±1.2 in the onion-treated group (Figure 3).

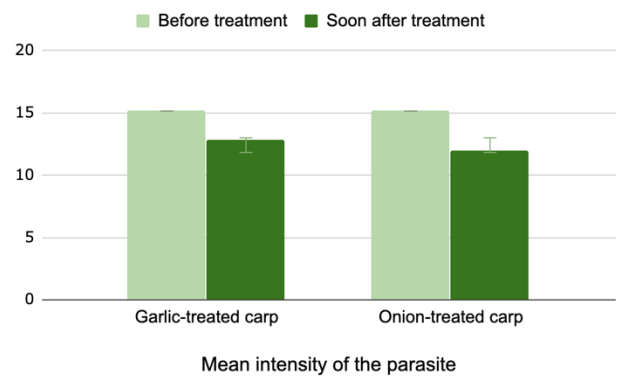


Figure 3. Reduction in mean intensity of *G. elegans* after 3 min exposure of carp to garlic and onion extracts.

DISCUSSION

Many plants and plant-derived compounds exert favorable effects to eradicate the disease agents with eliminating negative effects of synthetic chemical pharmaceuticals (Buchmann et al. 2003; Wu et al. 2011; Fridman et al. 2014; Wunderlich et al. 2017; Tavares-Dias 2018). The sensitivity of the plants against the diverse disease agents varies by the characteristics of the plant, indicating the need for more specific research (Trasvino Moreno et al. 2017; Hyun Kim et al. 2018; Tavares-Dias 2018). Various treatment effects of garlic on some Monogeneans have been studied in various fish species (Fridman et al. 2014; Schelkle et al. 2017) however, the studies on onion effects in fish parasites are relatively scarce (Yildiz et al. 2019). This study provides new information on the antiparasitic activity of onion against *G. elegans* (Monogenea).

In the present study, garlic and onion were evaluated for their antiparasitic activity against *G. elegans* found on the mucus of carp. Garlic and onion produce polar and apolar bioactive substances showing pharmacological effects such as cytotoxicity, antispasmodic and antifungal properties (Lanzotti 2012). Antiparasitic properties of main active compounds obtained from onion and garlic towards various parasite species have been reported (Corzo-Martínez et al. 2007). In the *in vitro* test, garlic and onion showed anthelmintic activity against *G. elegans* (Monogenean) from the skin of *C. carpio* depending on the concentration and duration. The finding of dose-dependent killing activity of garlic against Monogeneans in this study is in agreement with previous research on different fish and parasite species by Buchman et al. 2003; Fridman et al. 2014; Millitz et al. 2013; Soares et al. 2017; Hyun Kim et al. 2018; Yildiz et al. 2019.

Considering EC50 values of garlic and onion extracts, the most effective concentration for garlic was 8.37 ± 4.75 mg/mL 3 min and for onion 4.72 ± 7.10 mg/mL 3 min, revealing that onion was more effective against *G. elegans* than garlic. In our tests, in general, the survival time of *G. elegans* ranged from 1 min to 6 min in garlic-treated or onion-treated parasites. The findings of survival time ranges are similar to those reported in the previous study of Schelkle et al. (2013). However, Trasvino Moreno et al. (2017) has reported that the survival time of *Neobenedenia* sp. (Monogenea) exposed to garlic (dilution 1:10) was 7.6 h and 8 h for onion (dilution 1:10). The antiparasitic activity of garlic and garlic based material has been shown for a Ciliate, *Cryptocaryon irritans* *in vitro* however, *in vivo* tests with guppy, *Poecilia reticulata* infected with *C. irritans* were a failure to remove *C. irritans* (Hyun Kim et al. 2018). Garlic as the feed supplement decreased *Neobenedenia* sp. in barramundi, *Lates calcarifer* (Militz et al. 2013). The elimination of trichodinosis and gyrodactylosis from Nile tilapia, *Oreochromis niloticus* at a ratio of 68% has been reported by Abd El-Galil and Abdelhadid (2012). The intensity of *G. elegans* decreased by 14.40% in garlic-treated and 19.79% in onion-treated carp in the present study, showing lower efficacy levels than previous studies. Fridman et al (2014) reported that the prevalence of *G. turnbulli* decreased from 80% to 12.3% after bathing of infected guppy with garlic extract in the concentration of 7.5 mL/L for 1 h. Schelkle et al. (2013) revealed that garlic in the minced and granule forms reduced *G. turnbulli* by 66 and 75% after 3 subsequent applications and even 99% efficiency in Chinese freeze-dried garlic and allyl disulfide after a single treatment. Lower values of the reduction in parasite intensity here may be related with form of the garlic or onion used and the single application. Antiparasitic activity of the plants tested in the present study was higher *in vitro* tests than *in vivo* treatments. *In vivo* experiments were not with the similar results of *in vitro* tests as assessed by EC50. This can be explained by the penetration ability of monogeneans under scales as stated by Trujillo-González et al. (2015) or embedding into skin mucus. Fish epithelium can block the contact of allicin with the parasite, resulting in a decrease in efficacy (Hyun Kim et al. 2018). The repeated immersions may be necessary to increase the efficacy of the treatment to increase the probability of the contacts between parasite and the effective agents of garlic or onion. Unless the main active compounds of the plant such as allicin can activate apoptosis with reaching the parasite or disrupt the essential enzyme system in the parasite the efficacy of the immersions would not be high (Gruhlke et al. 2010).

Treatment of carp with garlic at the concentration of 8.37 mg/mL for 3 min or onion at the concentration of 4.72 mg/mL for 3 min caused slight changes of the parameters tested in general. Plasma glucose and lactate are reliable indicators of stress in fish as the changes in carbohydrate mechanism may occur under stress as reflected by higher levels of glycemia and plasma lactate (Pickering et al. 1982; Roque et al. 2010). Plasma glucose as an indicator of stress response increased after the treatments with garlic or onion in the present study. This can be explained by a fast response activated by the sympatho-chromaffin axis to provide glucose supply to critical tissues to overcome the stress (Balasch and Tort

2019). In short-term acute stress response, it is expected higher values of plasma glucose, exceeding 200 mg/mL (Yildiz and Pulatsu 1999). Plasma glucose values of garlic-treated or onion-treated carp did not show extremely high values and returned to control values after 1 h recovery. Plasma lactate values were similar to the control. Hematocrit values can be higher in stressful conditions to increase oxygen to the organs in response to higher metabolic demand (Cnaani et al. 2004). In this study, treatment with onion did not have any effect on hematocrit. The increase of hematocrit in garlic-treated carp was transient as hematocrit level returned to normal level after 1 h. In the overall evaluation of physiological response to the treatments with garlic and onion, it can be concluded that acute stress response due to the treatments with garlic or onion extracts was not clear as the variations in stress parameters tested here showed slight changes and returned to control level following 1 h recovery. Fish exhibited abnormal behavior in immersion treatment with garlic or onion, indicating behavioral stress response. Fish survival was not affected by garlic and onion treatment and the aquaponic system was operated without any problem. Although the efficacy of the garlic and onion treatment was low against the parasites the intensity of the parasites did not increase and the fish did not die due to parasites.

CONCLUSIONS

Parasitic diseases can be devastating to fish in an aquaponic system. Due to the complexity of the aquaponic system with the plant, fish and bacteria immersion or bath with non-toxic plant-derived substances may provide an alternative solution against pathogens. Garlic and onion showed antiparasitic activity against Gyrodactylidae (Monogenea) to some extent. *In vitro* antiparasitic activity of garlic and onion were dose-dependent to *G. elegans*. Based on EC50 values found in *in vitro* tests and subsequent *in vivo* tests, onion was more effective than garlic against *G. elegans*. *In vivo* application of garlic or onion with short term immersion resulted in low efficacy to *G. elegans* infection in carp, indicating the necessity of further investigations for more efficient forms of treatments in fish. The variations in the stress markers of carp exposed to garlic or onion may be attributed to mild stress response. The normal levels of hematocrit, plasma glucose and lactate after recovery in freshwater confirm the temporary stress effect of treatments with garlic or onion extracts. Garlic and onion can be considered as constituting antiparasitic potential to Monogeneans in fish despite their low efficiency. It is noteworthy that the use of garlic or onion to reduce parasite intensity in aquaponic systems is coherent for the context of eco-efficient aquaponic food production as the garlic and onion are non-toxic to human.

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