Effects of garlic (*Allium sativum*) and chamomile (*Matricaria chamomilla*) extracts on *Ichthyophthirius multifiliis* parasite in guppy fish (*Poecilia reticulata*)

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Abstract

The ornamental fish sector is considered a large international market and parasitical diseases cause serious damage to this trade's profits. This study was conducted to investigate the use of garlic (*Allium sativum*) and chamomile (*Matricaria chamomilla*) aqueous extracts on suppression of *Ichthyophthirius multifiliis* parasite in guppy fish (*Poecilia reticulata*). Two experimental groups consisted of T_1 (0.1 g/L garlic aqueous extract), T_2 (0.4 g/L chamomile aqueous extract), and control obtained. For each group, three replicates were prepared. The fish were exposed to parasite tomonts for 48h in a dark condition and then placed in a long-term bath with the mentioned herbal aqueous extracts for 14 days. At the end of the experiment period, the growth parameters, survival rate, parasite condition, and histopathological changes were investigated. No significant difference was observed in growth parameters. However, the survival rate in T_2 was higher than in T_1 and the control. The use of garlic extracts after four days and chamomile extract after six days as a long-term bath completely cured the parasitic issue in guppy fish. The histopathological study showed significant changes in the liver and gills of experimental fish. However, no histopathological change was observed in the control group. According to this study, garlic and chamomile had an effective influence on *I. multifiliis* parasite. These two herbs have reduced mortality rate and improved the guppy fish health and can be considered as alternative medicine and safe treatment in comparison with chemical solutions.

Keywords: Parasite, *Matricaria chamomilla, Allium sativum, Poecilia reticulata,* Histopathology.

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Introduction

Today the ornamental fish sector is considered a large international market. Besides direct losses caused by mortality, parasites may have a considerable impact on the growth, behavior of ornamental fish, resistance to stressing factors, and susceptibility to predation. The presence of parasites on ornamental fish bodies may also reduce marketability (Crowden and Boom, 1980; Brassard *et al.*, 1982). The ciliate protozoan *Ichthyophthirius multifiliis* Fouquet, 1876, "Ich" or white spot parasite is recognized to be one of the most pathogenic diseases of wild and cultured freshwater fish, especially ornamental ones. This disease is a critical problem for aquarists and commercial fish producers worldwide. While many protozoans reproduce by simple division, a single Ich organism can multiply into hundreds of new parasites (Fig. 1). Ich is capable of causing massive mortality within a short time after infestation. The technique to reduce the mortality rate of fish is a serious issue of fish culture (Swain and Nayak, 2009). Guppy fish is one of the hard species for aquarium hobbyists who are beginners. Many strategies were obtained for better farming of ornamental fish including the use of chemical therapeutics; however, each strategy besides useful aspects may have some side effects, so finding a strategy with the minimum side effect on fish, water, and the environment always is important. There are several pesticides commonly used to treat parasitic fish. However, the use of such pesticides in the aquarium is not applicable and could affect the environment and other organisms as well. There are several alternatives for treating parasites such as antibiotics, probiotics and prebiotics, each with a special application method and specific period. One of the reasonable solutions to solve the disease issues in fish farming is the use of plant extracts (Yildiz *et al.*, 2019).

The findings of the effects of herbal substances on fish parasites have been studied before (Picon-Camacho *et al.*, 2012; Erguig *et al.*, 2015; Tavares-Dias, 2018; Yildiz *et al.*, 2019). Anti-parasitic effects of garlic (Wunderlich *et al.*, 2017; Hyun Kim *et al.*, 2019) and chamomile (Gholipour-Kanani *et al.*, 2012) have been studied before as well. But there are lots of issues that must be considered. The side effects of these herbal medicines must be considered. This issue could be a critical one in the massive application of these herbs. For this aim, we carried out an in-vitro test to evaluate the antiparasitic effect of garlic and chamomile aqueous extracts on *I. multifiliis* in guppy fish. During this study, the growth, survival, parasite treatment and histopathological changes of the liver and gills of fish were studied.

Material and methods

Preparation of garlic and chamomile aqueous extracts

Dry garlic *A. sativum* and dried chamomile, *M. chamomilla* were obtained from a commercial market (Tehran, Iran). Garlic cloves were peeled and sliced into 1cm thick slices. Dried chamomile was powered manually. The obtained garlic slices with a concentration of 0.1g/L and chamomile with a concentration of 0.4g/L were stewed in 300mL of distilled water for 20 minutes and then the volume increased to 1000mL after Kazemipour *et al.* (2005). The prepared aqueous extracts were filtered through the Whatman filter paper and kept at 4˚C for use.

Fish maintenance and study setup

The total number of 90 parasite-free guppy fish with an initial body weight of 300.36±21mg was obtained from a local ornamental fish farm (Tehran-Iran). They were maintained in an independent plastic tank with a capacity of 50L until used for experimenting. After the adoption of fish to the experiment place for a week, they were randomly distributed into nine tanks with a density of 10 fish in each tank (three groups, each with three replicates, namely T_1, T_2 and Control, Fig. 1). All the tanks were maintained at 27±1ºC with permanent aeration. Fish got natural daylight and the experiment was carried out in a calm condition to reduce any stress. Fish fed on a commercial diet (47.5% crude protein, 6% crude fat) according to 10% of body weight three times daily. Fish from each treatment were exposed toparasite tomonts and kept in a dark condition for 48h post-challenge until parasitic infestation. The long-term bath with herbal extract was applied and everyday 50% of the rearing water was renewed. Every two days new herbal extract with a certain concentration was added. The fish wereobserved for any clinical signs and probably mortality for 14 days.

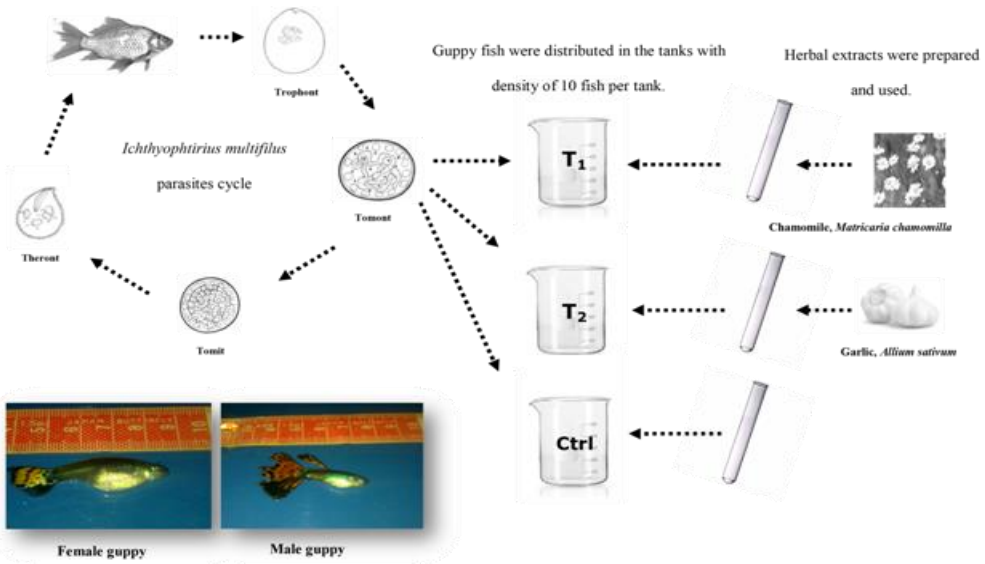

Figure 1: The schematic *I. multifiilis* parasite cycle and the experiment procedure.

Collection of Ichthyophthirius multifiliis tomonts

Fish with a natural heavy parasitic infestation (5 days post-infection) were collected from infected tanks of an ornamental fish store. The collected fish were anesthetized with the extraction of *Eugenia caryophlyllata* (100mg/L) and then washed with autoclaved tap water. The body surface of each infected fish was scraped to dislodge the tomonts. The isolated tomonts were concentrated with 70µm mesh. The collected tomonts were stocked in a glass tank filled with1L autoclaved tap water for parasitic exposure after Noe and Dickerson (1995).

Determination of growth performance and survival rate

The following growth parameters after experiment time were measured, weight gain (WG), specific growth rate (SGR), food conversion ratio (FCR), food conversion efficiency (FCE) and survival rate at the end of the experiment based on standard formulae as followed:

WG = Final weight – initial weight (Tacon, 1984)
SGR = [(LnFBW – LnIBW) / (t_1–t_0)] ×100 (Helland *et al.*, 1996)
FCR = intake feed / weight gain (Helland *et al.*, 1996)
FCE = weight gain/intake feed (Jafaryan, 2006)
Survival rate = [(N_0-N_t)/N_0] ×100 (Felix and Sudharsan, 2004)
Where, W=fish weight (wet weight, g); FBW=final body weight (g); IBW=initial body weight (g); t_1–t_0=experiment's duration

Determination of histopathological changes

A total of 18 guppy fish were collected from experimental groups and the control (six fish from each group). Gill and liver samples were dissected out and fixed in 10% buffered formalin embedded in paraffin and stained with hematoxylin and eosin (H&E) for optical examination. Histopathological sections were stained for general morphological purposes with H&E and viewed using the Olympus BX61 light microscope (Japan) and Olympus DP50 camera (Najdegerami *et al.*, 2016).

Statistical analysis

Treatments were compared by One-way Analysis of Variance (ANOVA) and Nonparametric tests (Chi-square). In a completely randomized design; comparisons of means were made using Duncan's multiple range tests using SPSS (Version 9.0). The significant level was set at $p<0.05$.

Result

Evaluated growth parameters are presented in Table 1. Group T_2 showed the highest WG, FCR, SGR, and FCE however they were not significant with T_1 and control ($p>0.05$).

Table 1: Some growth parameters of Guppy fish after 14 days of herbal extract treatment.

Parameters	Ctrl	Experimental groups	
		T_1 (garlic extract)	**T_2 (chamomile extract)**
Final weigh (mg)	345.42±3.99^{NS}	339.58±5.44 ^{NS}	350.52±5.96 ^{NS}
WG (mg)	45.12±2.91 ^{NS}	39.58±5.43 ^{NS}	50.52±5.9 ^{NS}
FCR	1.03±0.14 ^{NS}	1.5±0.12 ^{NS}	1.02±0.17 ^{NS}
SGR	0.99±0.08 ^{NS}	0.86±0.11 ^{NS}	1.09±0.12 ^{NS}
FCE	1.32±0.1 ^{NS}	1.16±0.16 ^{NS}	1.49±0.17 ^{NS}

Values expressed in mean ± SE, Different uppercase letters in the same row indicate significant differences, NS refers to no significant difference (n= 90, $p<0.05$).

The survival rate is presented in Figure 2. The highest survival rate was observed in the T_1 group that was treated with garlic extract (0.2g/L). Group T_2 which was treated with chamomile extract (0.4g/L) showed the lowest survival rate ($p<0.05$).

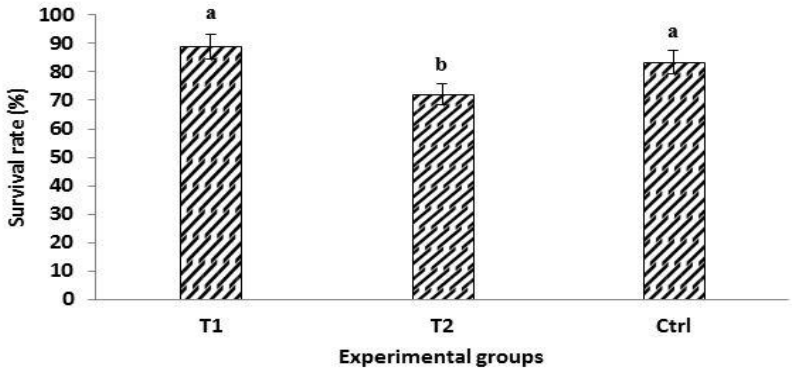

Figure 2: The survival rate of guppy fish over 14 days of long-term bath with garlic and chamomile aqueous extracts.

The result of parasitical evaluation after guppy fish exposure to *I. multifiliis* over 14 days is presented in Table 2 and Figure 3. Garlic extract after 4 days and chamomile after 6 days of a long-term bath successfully treated the *I. multifiliis* parasite in guppy fish. The normal morphology of gill filaments and lamellae in control fish is shown in Figure 4-A1.

Table 2: The presence of *I. multifiliis* on guppy fish over 14 days of long-term bath with garlic and chamomile extracts.

Treatments	Experiment days													
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th
T_1	+	+	+	+	-	-	-	-	-	-	-	-	-	-
T2	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Ctrl	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ show the presence of parasites, - show the absence of parasites, T_1, treated with garlic, T_2, treated with chamomile, Ctrl, control group.

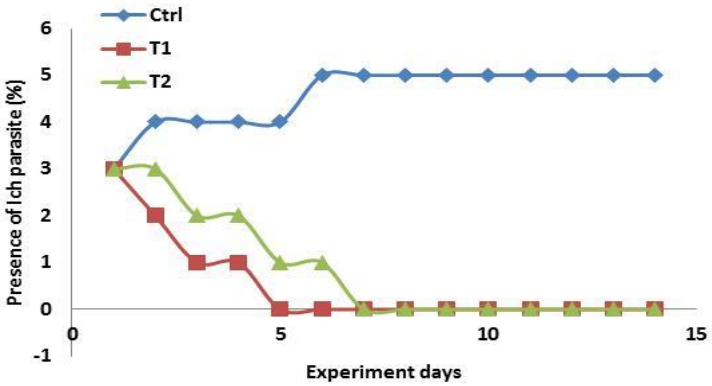

Figure 3: The percentage of *I. multifiliis* on guppy fish over 14 days of the experiment. Data are expressed with the Braun-Blanquet cover scale as follows, very few parasites, the cover is less than 1% (+); Many parasites, but the cover is 1-5% (1); Very many parasites or cover is 6-25% (2); Any number of parasites, the cover is 26-50% (3); Any number of parasites, the cover is 51-75% (4) and Cover is greater than 75% (5).

The lamellae are regularly lined up along both sides of a filament. The lamellae are covered by a one-cell thick lamellar epithelium and supported by pillar cells, which are contractile and separate neighboring lamellar capillaries. After guppy fish exposure to garlic and chamomile aqueous extracts, fish were examined for histopathological changes. The observed findings in the gills of both experimental treatments included expanded cartilaginous tissue, epithelial hyperplasia, increased space between filaments, Severe epithelial lifting in secondary lamellae associated with remarkable interstitial edema, reduced length of primary lamellae with severe degeneration in secondary lamellae, severe lamellar fusion, increased space between filaments associated with secondary lamellae degeneration, blood congestion and vasodilatation (Figs.4 to 6).

In the T_2 (chamomile extract) telangiectasia was observed (Fig. 7).

The use of garlic and chamomile aqueous extracts caused histopathological changes to appear in liver cells. As it is presented in Figure 8 the cytoplasm and vacuolar degeneration, nuclear pyknosis, hepatic necrosis and macrophage aggregation were recorded.

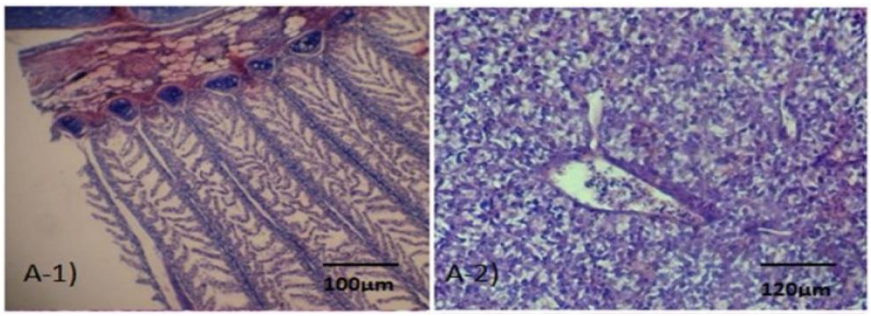

Figure 4: Light microscopic presentation from lams number 1 (control). gills (A-1), liver (A-2), H&E staining, A-1, 100X.

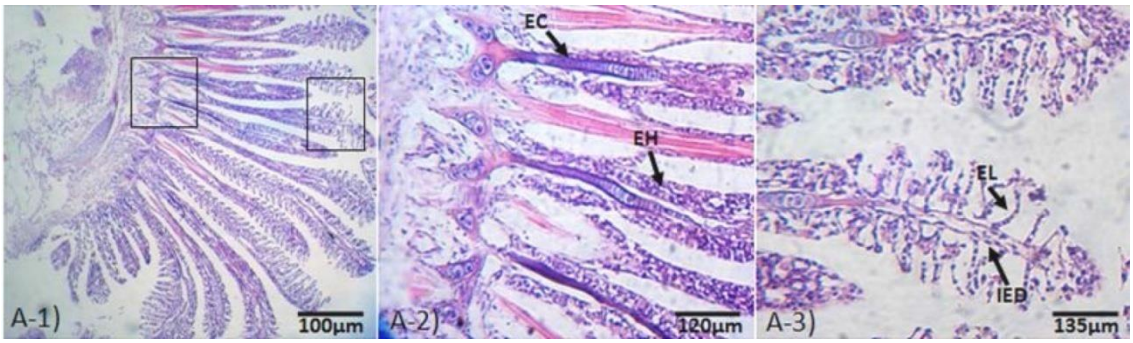

Figure 5: Light microscopic presentation from T_1 and T_2; See different lengths for primary lamellae in figure (A-1), The inserts present higher magnifications. (A-2), Note the expanded cartilaginous (EC) tissue and epithelial hyperplasia (EH) and increased space between filaments. (A-3), Severe epithelial lifting (EL) in secondary lamellae associated with remarkable interstitial edema (IED). H&E staining, A-1, 100X, A-2,400, A-3, 600X.

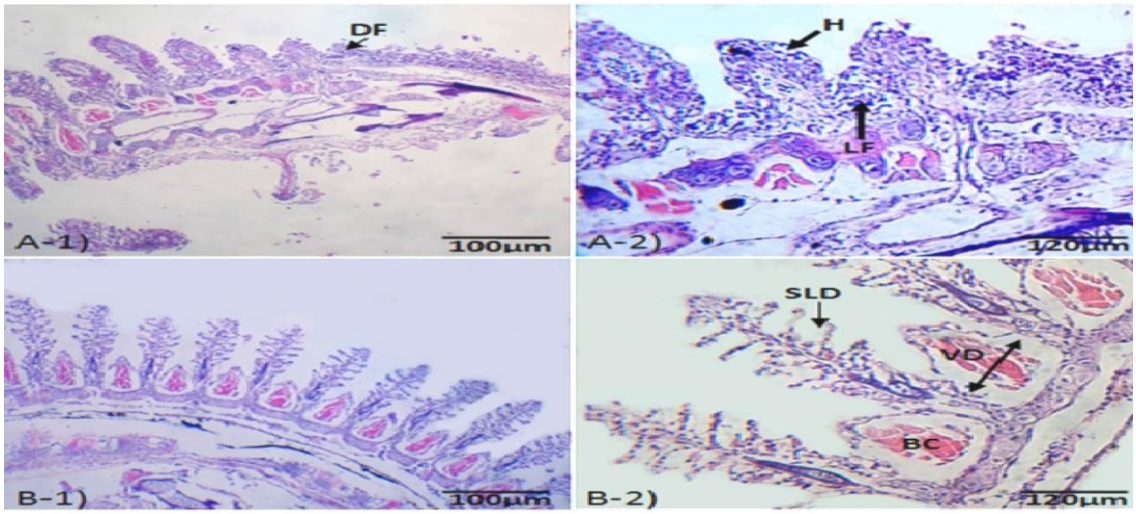

Figure 6: Light microscopic presentation from T1 and T2; (A-1), See the reduced length of primary lamellae with severe degeneration in secondary lamellae, note the higher magnification presented in (A-2), The gills are presented with epithelial hyperplasia (H) and severe lamellar fusion (LF). (B-1), Shows increased space between filaments associated with secondary lamellae degeneration (SLD), blood congestion (BC), and vasodilatation (VD) in higher magnification (B-2). H&E staining, A-1 and B-1, 100X; A-2 and B-2, 400X.

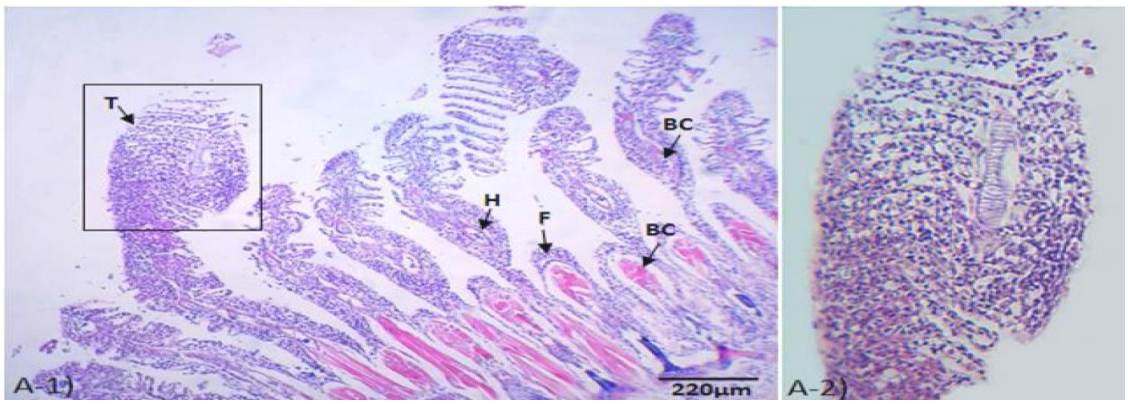

Figure 7: Light microscopic presentation from T2, (A-1), The gills are presented with epithelial hyperplasia (H), severe lamellar fusion (F), blood congestion (BC), and vasodilatation, see the insert section for telangiectasia (T) in higher magnification (A-2). H&E staining, A-1, 100; A-2, 600X.

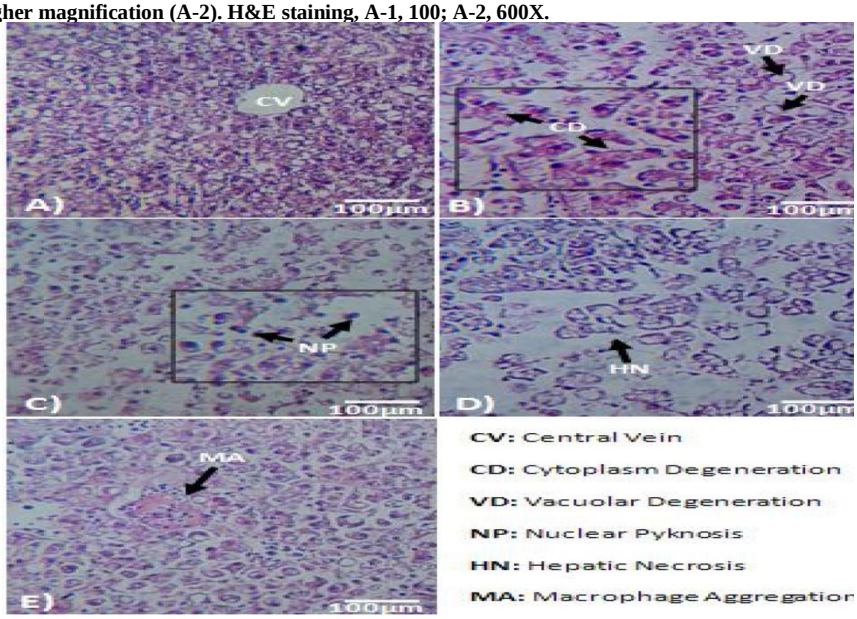

Figure 8: Cross-section of the liver in different Lams. (A), Ctrl, (B) T1 and T2, (C), T1 and T2, (D) T2 (E) Ctrl, T1, and T2. H&E staining, 400X.

Discussion

Ichthyophthirius multifiliis, the causative agent of white spot diseases, is an aggressive parasite, infesting a wide range of freshwater fish species and causing significant mortality. Several treatment methods consisting of pesticides or chemical substances are restricted in some countries. Herbal medicine can be considered a suitable alternative method for treating parasitical issues. This type of medicine may have several effects on recipient fish and some of these effects are still unknown. Garlic and chamomile are two well-known herbs that have been used in traditional medicine for centuries (Kazemipour *et al.*, 2005). Fish growth is affected by many factors like the feeding period, kind of diet, and environmental factors. Herbal medicine may affect fish growth as well. The insignificant results of growth parameters in our study might be because of the short period of the experiment. In our study that lasted for 14 days, we first challenged guppy fish in the first 2 days before the experiment with Ich tomonts. The results demonstrated that the use of these two herbal extracts has no significant effect on growth parameters, however, the T_2 showed the highest score (p>0.05). In general antimicrobial effects of garlic and chamomile are supposed to be effective on the microflora of the host fish alimentary canal and this could help to improve the feed utilization and retention and finally cause significant growth (Khalil*et al.*, 2001). The insignificant results of growth parameters in our study might be because of the short period of the experiment. Our finding is in line with those of Gholipour-Kanani *et al.* (2012), that used the same herbal medicine with the same concentration in treating Sail-fin molly fish, *Poecilia latipinna*. In another study, Aly *et al.* (2008) reported that 2 months of dietary feeding of garlic didn't cause any significant effect on the growth parameters of Nile tilapia, *Oreochromis niloticus*.

The development of different disinfecting agents to treat parasites is one of the most important aspects of aquatic animal health and diseases. Garlic could be compared with antibiotic agents that can control pathogens, such as bacteria and fungi (Adetumbi *et al.*, 1986; Ress *et al.*, 1993; Coroz *et al.*, 2007). It has been considered that garlic, *A. sativum* has several beneficial effects on humans and animals, exhibiting antimicrobial, antioxidant, and antihypertensive properties (Konjufca *et al.*, 1997; Sivam, 2001, Sivaram *et al.*, 2004). There are many studies on garlic products and their effects on the treatment of diseases (Kazemipour *et al.*, 2005; Gholipour-Kanani *et al.*, 2012; Karimi Pashaki *et al.*, 2020), however; there are very few reports about garlic effects on ectoparasites. In our study garlic extract with a concentration of 0.2g/L after 4 days of long-term bath inhibited the Ich parasites and after that no longer the parasite was observed. The other herb that was used in this study was chamomile which successfully suppressed the Ich after 6 days of the long-term bath. Kazemipour *et al.* (2005) reported that chamomile aqueous extract could treat ulcers in common carp (*Cyprinus carpio*). The Ich parasite is sticking to the host fish's body surface and uses the body fluids. The chamomile extract besides the inhibition of the Ich parasite could speed up the injured parts' remedy in infected fish (Kazemipour *et al.*, 2005). A similar result was reported by Gholipour-Kanani *et al.* (2012) that after 5 days of treatment with garlic and chamomile, the Ich parasite completely disappeared. This finding may be due to the effective components that can be found in these two herbs. The treatment of this parasite in guppy fish as well causes an improvement in survival rate in guppy fish. A similar finding was reported by Kim *et al.* (2001) and Jain and Wu (2003). In our study, the survival rate was significantly greater in T1 (garlic treatment) when compared to the control.

The treatment of Ich which is causing mass mortality in fish would be the causative of the high survival rate in treated guppy fish. Garlic and chamomile aqueous extracts with rapid treatment time showed promise for further development into a proactive management tool to eradicate or weaken *I. multifiliis*.

The study of the histology of fish has been performed for a better understanding of the body's internal changes, which are caused by chemicals or biological agents (Camargo and Martinez, 2007). The inclusion of cells within the organs like gills, kidneys, spleen, and liver is involved in fish health characteristics. In our study the use of garlic and chamomile extract in a long-term bath in guppy fish, *P. reticulata* caused significant changes in the liver and gills' structure. It has been suggested that garlic would be an ideal chemical for bathing the infested fish and this is because of the rapid biodegradation of garlic after use. The selective toxicity of garlic makes it relatively harmless to aquaculture stock (Lee and Gao, 2012). In our study gills and liver were examined for histopathological changes. The characteristics of the exterior structure of the gills in fish adjusted to the gills' function of improving the efficiency of gas exchange and increasing the exchange surface area (Guan and Lin, 2004). Because the gills are the main site for gas exchange and other important functions such as ionic and osmotic regulation and acid-base equilibrium, histopathological changes in the structure of the gills involve respiratory disturbances and electrolyte imbalance (Cerquira and Fernandes, 2002). The normal morphology of gill filaments and lamellae in control fish was observed (Fig. 4-A1). The lamellae are regularly lined up along both sides of a filament. The lamellae are covered by a one-cell thick lamellar epithelium and supported by pillar cells, which are contractile and separate neighboring lamellar capillaries. After guppy fish exposure to garlic (T_1) and chamomile (T_2) for 14 days, histopathological changes were observed in the gills of both treatments (Figs. 4 and 5). Agbebi *et al.* (2013) confirmed the use of dietary garlic in-feed could cause histopathological changes in the gills' structure. Any changes in gills structure like what was observed in our findings, including epithelial hyperplasia, and severe lamellar fusion in gills can cause major changes in osmotic balance and the oxygen level carried by red blood cells (Pleuranen *et al.*, 1994). The blood vessel dilation in gills that was observed in experimental treatments might be happening for increasing the blood circulation in gills to increase its performance. The liver of fish is an important organ of active metabolism and detoxification, and is extremely sensitive to the pollutant. Extraneous xenobiotic compounds biotransformation occurs in the liver (Brusle and Anadon, 1996; Carmago and Martinez, 2007). Histopathological changes were observed in the liver of both treatments (T_1 and T_2) which included cytoplasm degeneration, vacuolar degeneration, and nuclear pyknosis (Figure 6- B, C). The use of 20% of garlic in the feed of African catfish, *Clarias gariepinus*, causes vascular degeneration in hepatocytes (Agbebi *et al.*, 2013). In T_2 treated with a chamomile long-term bath hepatic necrosis was observed (Fig. 6- D). Hepatic necrosis resembles toxic injury to the liver with sudden onset that can reduce the liver's function. The use of herbs with high concentrations might be a reason for necrosis in the liver. The liver is the first place that the external substances would reach, so any substance from outside would affect the liver directly. Similar to our result Al-Salahy and Mahmoud (2003) reported that livers of fish administered garlic for 11 days showed dilatation of some blood vessels. Also, Bhati *et al.* (1973) reported that liver damage elevates enzymatic levels in blood under certain pathological conditions. This could affect the host organism's metabolism like the reduction of

food retention. El-Barbary *et al.* (2018) reported that garlic juice, (20g/kg body weight), caused cytoplasmic vacuolation with dilation, vacuolar degeneration and congestion in blood vessels of the liver cells of African catfish, *Chrysichthys auratus* which was in the same line with our study (Fig. 7). Additionally, hepatic necrosis was also observed in guppy fish treated with chamomile and it was similar to Camargo and Martinez (2007) (Fig. 7-D). Macrophage aggregation was observed in all treatments including control (Fig. 8) and this might be because of *I. multifiliis* infestation. Herbs are the most accessible medicine which can be used in the aquaculture industry to reduce chemical material. According to this study, garlic and chamomile had an effective influence on *I. multifiliis* parasite. These two herbs have reduced the mortality rate and improved guppy fish health. Besides these findings, working on different concentrations and the mixture of these two herbs for future study is considerable.

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