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Original Article

In vitro Study of Effects of Alcoholic Extract of Pomegranate Peel on *Ichthyophthirius multifiliis* Theronts

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ABSTRACT

The *Ichthyophthirius multifiliis* (Ich) is one of the most important parasites in aquaculture industry, which causes the fatal disease of Ichthyophthiriasis. Treatments for this parasite include the use of certain chemical medications. However, due to the many negative effects of chemical compounds on the environment and humans, extensive studies of plant extract effectiveness in treating this parasite are very important. This study evaluated the alcoholic pomegranate (*Punica granatum*) peel extract's antiparasitic activity against the parasite *I. multifiliis* in vitro. Under laboratory conditions, the anti-parasitic efficacy of pomegranate peel alcoholic extract against *I. multifiliis* at doses of (0.5, 1, 2, 4, and 8 g/l) was evaluated after exposure for six hours. Additionally, this extract's toxicity was assessed for 96 hours on zebrafish at dosages of 0.5, 1, 2 and 4 g/l. The collected data were statistically contrasted with the results of the positive control sample (15 ppm formalin) and the negative control treatment. The study revealed that the theronts may be destroyed in 6 hours by concentrations of 2, 4, and 8 g/l, in addition to the concentration of 1 g/l, which caused the death of 96% of theronts within this period. In the toxicity test, the concentrations (2, 4, and 8 g/l) were highly toxic, and all the fish died, but the concentrations of 1 and 0.5 g/l were safe doses within 96 hours. Also, the value of EC_{50} in this research was calculated as 1.41 g/l. Therefore, the alcoholic extract of pomegranate peel in doses of 2, 4, and 8 g/l is not effective for clinical treatment and is only suitable as an antiseptic. So, the dose of 1 g/l has very good results and is recommended for clinical treatment.

Running Title: Effects of alcoholic extract of pomegranate peel on *I. multifiliis*

INTRODUCTION

The aquaculture industry's most important sectors are food and ornamental fish cultivation. Aquaculture has emerged as a rapidly expanding food-production technology on a global scale. [1-7]. The parasite *Ichthyophthirius multifiliis* is an obligate holotrich parasite whose size can reach one millimeter and is the cause of ichthyophthiriasis. This parasite is in the *Oligohymenophora* class, the *Hymenostomata* subclass, the *Heminostomatida* order, and the *Ichthyophthrididae* family [8-12]. This parasite can infect freshwater fish (both farmed and wild fish) at all ages, and depending on the level of contamination, it can cause up to 100% mortality. The wide spread of this disease can be due to the displacement of the infected host, the wide host range, and the ability of the parasite to multiply rapidly [13-17] Ichthyophthiriasis is also known as "white spot disease" since the primary sign of the illness is the development of white spots on the host's body. This parasite is one of the most important infectious agents in the ornamental and edible fish industry (especially salmon), and every year it causes great damage to the aquaculture industry worldwide [18]. The life cycle of this parasite consists of three stages (in order: theront, trophont, and tomont); the first stage swims freely in the water; the second stage is on the body of the fish; and the third stage is the reproductive form of this parasite [15]. The environment-adaptability of this parasite, its life cycle's reliance on temperature, its hiding place in the host's epithelium (where it hides from potentially fatal substances and medications), and the formation of

cysts around tomont are some of the factors that make treatment for this disease challenging [15, 19]. It is said that the most sensitive stage of this parasite's life to treatment is the first stage of its life, or the infectious theront [15]. This parasite attacks the epithelium of the skin and gills of fish, and it disrupts the function of the respiratory system and the excretory system of fish [20]. Until now, various chemical compounds have been introduced to treat the *I. multifiliis* parasite. Among these, green malachite, formaldehyde, chloramine-T, and copper sulfate can be mentioned [19]. However, each of these has its drawbacks. For example, green malachite (despite its excellent solubility in water and its effect on all life stages of this parasite) has been prohibited due to its carcinogenic nature and accumulation in fish tissue [20- 22]. Treatment with formaldehyde should also be done with high repetition to create a good result. In addition, formaldehyde reduces oxygen in the environment. Repeated treatment with copper sulfate can also be toxic for fish [23]. High doses of Chloramine-T can cause damage to the epithelial tissue of fish, it should be used with caution. The use of chemicals in the treatment of this parasite can cause environmental damage and pose risks to human health. For this reason, there is an urgent need for effective and safe alternative agents to fight this parasite. Therefore, plant compounds due to their degradability in nature, the absence of drug resistance, and their good effectiveness can be a suitable alternative to chemical compounds [24]. Garlic extract (*Allium sativum L.*) had promising effects on this parasite at the laboratory level [25]. Among the plant extracts that are effective against the parasite are papaya seeds (*Carica papaya*), green velvet bean leaves (*Mucuna pruriens*) [26], *Maceleaya cordata* extract [27], The combination of garlic and chamomile extract [28], aqueous extract of *Capsicum frutescens* [29], ethanolic extract of *magnolia officinalis* and *Sophora alopecuroides* [30], methanolic extract of *Psoralea corylifolia* [31] and *Morus alba* acetone and ethyl acetate extract [32] as well as compounds extracted from plant extracts such as dihydrosanguinarine and dihydrochelerythrine [33] Pentagalloylglucose [34], chelerythrine and chloroxylonine [35], cynatratoside-C [36] 20014b) and 10-gingerol [37]. Effects of tannic acid and alcoholic extract of *Chelidonium majus* L. and *Zataria multiflora* on *Ichthyophthirius multifiliis* have been studied in Iran [38, 39, 40, 41]. The pomegranate (*Punica granatum*) is a plant belonging to the *Punicaceae* family. This family includes one genus and two species (*Punica granatum* and *Punica protopunica*). The first species is edible and native to Iran and the Mediterranean region, and the second non-edible species is native to the Socotra islands. Metabolites in different parts of pomegranate fruit include sugars, organic acids, alkaloids, polyphenols, flavonoids, anthocyanins, fatty acids, and vitamins. The pomegranate peel's aqueous and methanolic extracts exhibit superior antibacterial qualities to both the water and the core. This may be because they include higher concentrations of tannins and phenolic chemicals [42]. In general, there is a direct relationship between phenolic compounds and antioxidant activity and antimicrobial properties. All parts of pomegranate have a lot of tannins, which has a relatively strong astringent effect. Pomegranate flower extract or decoction is effective for simple diarrhea, dysentery, upset stomach, and mucous secretions of the genital tract [43]. The pomegranate root bark is used fresh, dry, or in alcoholic extract due to the presence of alkaloid substances to eliminate intestinal worms [44]. Pomegranate juice has very good biological activity, such as anticancer [45], antibacterial [46], antifungal [47], anti-inflammatory [48], antioxidant activity [49]), strengthening the immune system [50], prevention of heart disease, liver fibrosis [51], and preventing lipid peroxidation even at lower concentrations than vitamin E [52]. The purpose of this research is to investigate the anti-parasitic effect of the alcoholic extract of pomegranate peel in vitro and in vivo on the parasite *Ichthyophthirius multifiliis*.

MATERIALS AND METHODS

preparation of Alcoholic Extract of Pomegranate Peel

The process of making an alcoholic extract from pomegranate peel involves washing the peel, allowing it to dry at room temperature, and then grinding it until it is completely powdered. Next, the powder is combined with a 1:5 ratio of 99.8% alcohol and left to soak for 20 hours. Then it was poured into the decanter container so that 1 cm was placed on the halal plant so that it does not dry, and cotton was also used as a strainer so that nothing enters the extract and it is transparent (in this case, the decanter valve is closed). After 24 hours, the decanter valve was opened so that the liquid or extract came out, and then the decanter valve was closed again and the solvent was poured on the plant inside the decanter. This cycle was repeated for 2 to 3 days until the plant turned from red to yellow

and the cotton was smooth to the original color, then the clear extract was placed in a rotary machine with a temperature of 55 degrees Celsius and 30 revolutions to separate the ethanol. The rotary device is connected to the vacuum pump. In the next step, pour the extract onto a large plate and place it in a 37°C incubator until it dries completely. With a sterilized razor, the dried sediment was scraped and gathered.

Evaluation of the Toxicity of the Alcoholic Extract of Pomegranate Peel in Zebrafish

To investigate the toxicity of pomegranate peel alcoholic extract, 160 zebrafish that appeared to be in good health were acquired from the ornamental fish breeding center. After that, they were moved to the University of Tehran, Faculty of Veterinary Medicine's Aquatic Research Center, where they were adapted to new tanks for 14 days. The fish's health was then confirmed by randomly inspecting ten of them for internal and external parasites. Then 150 fish were selected and divided into 5 groups, including 4 treatment groups with doses of 4, 2, 1, and 0.5 g/l and a control group (without extract). Each group included three replications, and 10 zebrafish were used in each replication. The experiment continued for 96 hours, and mortalities were recorded during this time.

Isolation of the Parasite and Preparation of Parasite Theronts

First, 10 fish infected with the parasite *I. multifiliis* were purchased from an ornamental fish store. Then, to isolate the trophonts, a wet smear was carefully prepared from the surface mucus of the body of the fish infected with adult trophonts, and all the contents were placed in a petri dish containing the aquarium water. Then, by pipetting, the trophonts were released into the water. After 3 to 5 days at 25 °C ambient temperature, infectious theronts with fast movements could be seen under the microscope. Also, the density of theronts reached 6000 per milliliter using a centrifuge (at 4000 rpm for 10 minutes). Thomas slide was also used to count the theronts [31].

Preparation of Extract Concentrations

Six distinct concentrations of pomegranate peel alcoholic extract (0.5, 1, 2, 4 and 8 g/l), formaldehyde concentration (15 mg/l, positive control), and a negative control group without disinfectant solution (containing chlorine-free aquarium water) were prepared following preliminary experiments and a review of sources:

Treatment 1: negative control containing chlorine-free aquarium water
Treatment2: positive control containing formalin with a concentration of 15 ml/l
Treatment 3: concentration of 8 g/l extract
Treatment 4: concentration of 4 g/l extract
Treatment 5: concentration of 2 g/l extract
Treatment 6: concentration of 1 g/l extract
Treatment 7: concentration of 0.5 g/l extract

Conditions of Exposure of the Theronts to Different Concentrations of Alcoholic extract of Pomegranate Peel

Initially, each group well received 50 microliters of theront suspension, or 600 theronts. Then, with a volume of 50 microliters, various concentrations of the pomegranate peel alcoholic extract were added to the parasite-containing wells, in accordance with the doses listed in the previous section. The parasite death rate was then computed at 2-, 4-, and 6 hours following exposure.

Calculating the Survival and Mortality Rates of Theronts

The following technique was used to calculate the number of deaths after the theronts' exposure period: In summary, 10 microliters of each well's contents were put on the Thomas slide and under the coverslip. Then, the number of live theronts was counted in nine large squares of the Thomas slide, and 10% was added to their number to obtain the number of live theronts per cubic millimeter. The transformation of the parasite theronts, which is brought on by cell lysis, was used to determine the death of the theronts.

Statistical Analysis

To calculate the effect of the alcoholic extract of pomegranate peel on the parasite, the data from each experiment was analyzed using one-way ANOVA and Duncan's test ($p \leq 0.05$) in SPSS software (version 25) [53]. Also, SPSS software (version 25) and the Probit method were used to calculate the toxicity of pomegranate skin extract on zebrafish [54].

RESULTS

The Effect of the Pomegranate Peel Extract on *Ichthyophthirius multifiliis* Theronts

Figures 1 to 3 are the results of the laboratory study of pomegranate peel extract in different concentrations (8, 4, 2, 1, and 0.5 grams per liter) at different test times (2, 4, and 6 hours). It shows the mortality rate of the theronts of the *Ichthyophthirius multifiliis*. The results of the study showed that the death rate of parasitic

theronts has a direct and significant relationship with the concentration of the compound and exposure time.

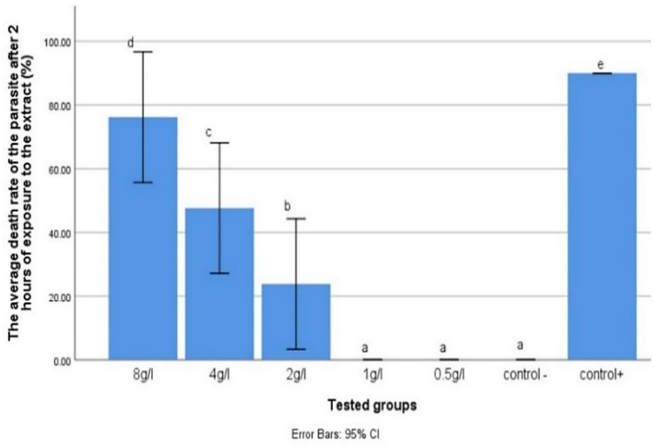

Fig. 1 Average parasite mortality rate after two hours of treatment with pomegranate peel extract (%). By increasing the dose of the extract, the death rate of the parasites also increases. After two hours, the doses of 1 and 0.5 g/l had no effect on the parasite. Other doses of the extract are significantly different from each other and from the positive and negative control groups. After two hours, formalin was able to kill more than 80% of the theronts population. The significance of differences between groups is indicated by letters.

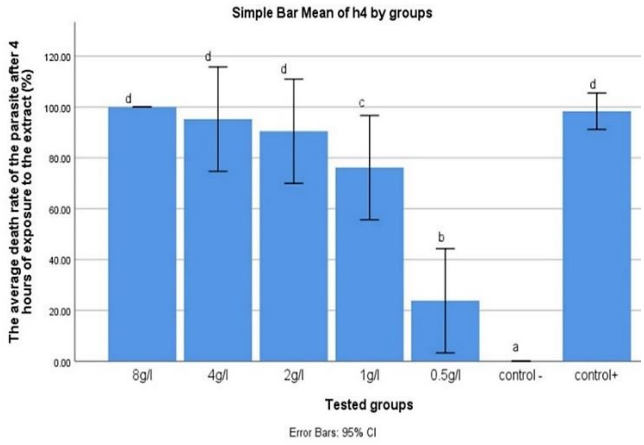

Fig. 2 Average parasite mortality rate after 4 hours of treatment with pomegranate peel extract (%). As shown in the figure, the doses of 8, 4, and 2 g/L killed more than 80% of the parasites after 4 hours, and there is no significant difference between each other and the positive control group. Also, the dose of 1 g/L has left a good performance, although it still has a significant difference with the positive control group and its higher doses. The significance of differences between groups is indicated by letters.

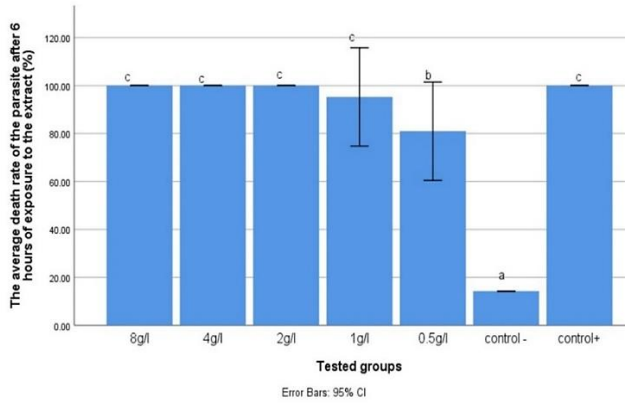

Fig. 3. Average parasite mortality rate after 6 hours of treatment with pomegranate peel extract (%). As shown in the figure, the doses of 8, 4, and 12 g/L after 6 hours are not significantly different from each other and the positive control group. Also, the dose of 0.5 g/L killed more than 80% of the population of theronts. Although it still has a significant difference from other groups, The significance of differences between groups is indicated by letters.

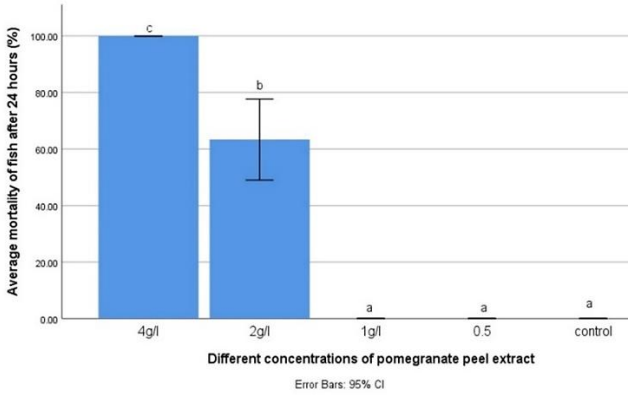

Fig. 4 The average mortality rate of fish exposed to different concentrations of the extract after 24 hours (%). As shown in the figure, the dose of 4 g/l caused the death of all fish after 24 hours. Also, the dose of 2 g/l has led to the death of more than 60% of fish. The significance of differences between groups is indicated by letters.

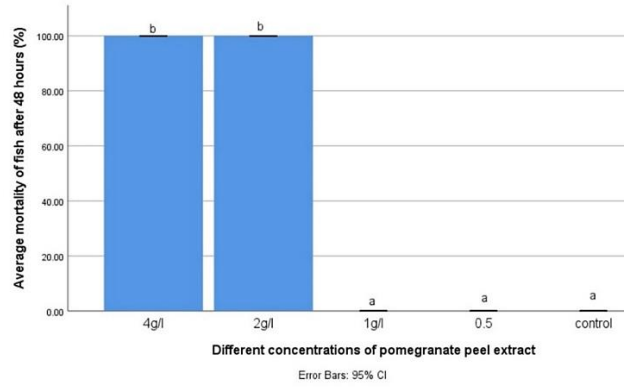

Fig. 5 The average death rate of fish exposed to different concentrations of the extract after 48 hours (%). As shown in the figure, the doses of 4 g/l and 2 g/l resulted in 100% fish death after 48 hours. No mortality was observed after 48 hours in 1 and 0.5 g/L groups. The result did not change until 96 hours after exposure to the extract. The significance of differences between groups is indicated by letters.

The shortest period to kill more than 80% of parasites is 4 hours, which corresponds to 2, 4, and 8 grams per liter. These doses were able to kill all the parasites in 4 hours. The doses of 0.5 and 1 gram per liter after two hours of exposure to the extract did not affect the parasite, and their performance was not significantly different from each other or the control group. After two hours, the 2 grams per liter dose performed significantly differently from other groups, and after four hours, it killed more than 80% of the parasites. Its performance after 4 hours with doses of 4 and 8 grams per liter and the positive control group had no significant difference. It should be noted that all four groups of 2, 4, 8 g/L and the positive control group (formaldehyde) caused the death of all parasites after 6 hours. The performance of the dose of 1 g/l has been different at different times, 2 hours after being exposed to the extract, it had no effect on the theronts, and in this respect, its lower dose (0.5 grams per liter) has no significant difference. After 4 hours, it was able to destroy more than 70% of parasites, and after 6 hours, more than 90% of parasites. The performance of this dose after 4 hours of exposure to all groups showed a significant difference, but after 6 hours, there was no significant difference with the higher doses. The performance of the dose of 0.5 g/l also improved, although there was no significant difference between the 1 g/l group and the control group after two hours of exposure. However, after 6 hours of exposure to the extract, it was able to destroy nearly 80% of the parasites. The performance of this dose after 6 hours of exposure to the extract showed a significant difference, with all the groups lower and higher. After 2 hours, the dose of 8 g/l destroyed more than 70% of the theronts and had a significant difference with all groups. This dose was able to increase the death rate of the parasite to 100% after 4 hours. After 2 hours, the dose of 4 grams per liter destroyed more than 40% of the parasites, and there was a significant difference between all groups.

Evaluation of the Toxicity of the Alcoholic Extract of Pomegranate Peel in Zebrafish (*Danio rerio*)

In this study, to calculate short-term toxicity in Zebrafish (*Danio rerio*), doses of 0.5, 1, 2, and 4 g/L were evaluated to investigate short-term toxicity in 96 hours. Doses were chosen based on the results obtained from exposure of theronts to different concentrations of the alcoholic extract of pomegranate peel. In this study, at a concentration of 4 grams per liter, the fish had 100% losses in the first 24 hours. At a concentration of 2 grams per liter, the rate of mortality in the first 24 hours was close to 60% and reached 100% by 48 hours after exposure to the alcoholic extract of pomegranate peel. In other investigated concentrations, no losses were observed until the end of 96 hours. (Fig. 4 and 5). Also, EC_{50} was calculated at 1.41 g/l.

DISCUSSION

Nowadays, due to high efficiency and low bio-environmental risks, studies on the use of compounds and extracts of medicinal plants to control some parasites, including *Ichthyophthirius multifiliis*, have received more attention than before. Liang *et al*. [55] investigated the effect of two extracted flavonoids (kuwanons G and O) from a type of plant with the scientific name *Morus alba*. Their results indicated a 100% lethality effect of these flavonoids on theronts. Yao *et al*. [33] showed that the use of an alkaloid called dihydrosanguinarin, in the concentration range of 5.18 to 9.43 mg/l, has a very effective lethality coefficient on the theronts in laboratory conditions. Their previous research [27] showed that different concentrations of sanguinarine extracted from a native plant in China, *Macleaya cordata*, had a significant killing ability on parasitic theronts under laboratory conditions.

Shan *et al*. [35] reported the strong lethal effect of two alkaloid compounds, including chelerythrine and chloroxylonine, on parasitic theronts in laboratory conditions. Shinn *et al*. [56] also showed that the use of a concentration of 1 mg/l of bronopol chemical compound for 12 hours could not kill all theronts, but it significantly reduced the infectivity of theronts. It has also been reported that the duration of exposure to low concentrations of bronopol led to a decrease in trophont numbers in rainbow trout [57].

In the study of Ekanem *et al*. [26], the effects of the crude methanol extract of green velvet bean leaves and papaya seeds on the Ich parasite under *in-vitro* and *in-vivo* conditions on goldfish (*Carassius auratus*) were evaluated. In vitro conditions, the concentration of 150 mg/l of bean leaf extract and 200 mg/l of papaya seed extract caused 100% death of parasites after 6 hours. Under *in vivo* conditions, parasite-infected fish were immersed in a bath with green bean extract for 72 hours and in a bath with papaya extract for 96 hours. After treatment with the extract of both plants at a concentration of 200 mg, a 90% decrease in the number of Ich parasites was observed compared to the control group.

Fu *et al*. [37] isolated three active compounds named 10-gingerol, 6-dehydroshogaol, and 6-dehydro-10-gingerol from the ginger plant and investigated the effect and mechanism of their antiparasitic activity on the Ich parasite in grass-eating carp. Among these three compounds, 10-gingerol has the most antiparasitic effect in laboratory conditions. So, in concentrations of 2, 8, and 16 mg/l, respectively, it causes 100% destruction of theronts, non-encapsulated tomonts, and encapsulated tomonts, which has this effect through increasing osmotic pressure and radical accumulation. It causes free radicals and membrane damage. The results of study of tannin-rich tropical plant *Lysiloma latisiliquum* on the population of adult parasites revealed that the use of this plant for a short period due to its high tannic acid content can directly affect the biology (size of parasitic worms and female fertility) of adult *Haemonchus contortus* [58].

The study of the antiparasitic effect of 30 plant species against the Ich parasite, showed that concentrations of 10 mg/L of the extracts of two plants, *Magnolia officinalis* and *Sophora alopecuroides*, can kill all Ich parasites after 3 and 4 hours under *in-vitro* conditions [30]. The result of this study was the same as the result of our experiment. Also, Buchman *et al*. [25] investigated the effect of garlic extract on the Ich parasite and showed that the concentration of 62.5 mg per liter killed all the parasites after 30 minutes. The result of this study was the same as the result of our experiment.

Comparative in vitro and in vivo effects of feed additives including garlic, coriander, oregano, thyme, and astaxanthin, as an oral antiparasitic agent in rainbow trout, have been studied [59]. Among these 5 plants, garlic has the highest killing effect on the parasite theront stage in an in vivo situation. After that, oregano, thyme, and astaxanthin have had the most effect, respectively, while coriander has not shown any anti-parasitic effect in laboratory conditions. Also, the immune response was investigated by measuring the activity of plasma lysozyme in this research, and it was shown that these additives cause a significant increase in the activity of plasma lysozyme. As a result, these additives directly and indirectly prevent the initial growth of the parasite by increasing the host's immune response [59]. Investigation of effects of the ethanolic extract of Shirazi thyme (*Zataria multiflora*) on the tomont and theront stages of *I. multifiliis* in zebrafish, concluded that 20 ml of Shirazi thyme extract in the time interval of 6.04 to 6.37 minutes and the concentration of 10 ml of the extract per liter can destroy all Ich theronts in a period of 2.31 to 32.4 minutes. The results of this research were in line with the current research. Also, the use of this extract significantly reduced the production and proliferation of tomonts and reduced the severity and prevalence of Ich infection [40]. Study of the anti-parasitic effect of tannic acid on ich theronts under *in vivo* and *in vitro* conditions at concentrations of 0.75-7 mg/l at different times of 1-3 hours have been performed [38, 60, 61]. Results of parasitic theronts mortality were similar to the results of the present study. It had a direct and significant relationship with the concentration of the compound, and the number of parasite deaths increased significantly with the increase in tannic acid concentration from 0 to 7 mg/l. Also, increasing the exposure time from 1 to 3 hours led to a significant decrease in parasite theronts [38, 60].

CONCLUSION

In this study, the effect of the alcoholic extract of pomegranate peel in different concentrations (0.5, 1, 2, 4 and 8 g/l) and at different times (2, 4, and 6 hours) under *in vivo* conditions on the death rate of the *Ichthyophthirius multifiliis* theronts was investigated. The mortality rate and the percentage of survival of parasitic theronts were different indicators determining the effect of parasiticides during the period. Another indicator to evaluate the potential of parasites is the time that it takes to kill all the parasites. The results of this study showed that the alcoholic extract of pomegranate peel has an antiparasitic effect on *Ichthyophthirius multifiliis* under *in vivo* conditions. By increasing the concentration and exposure time of this extract, a significant increase in the anti-parasitic properties of this extract was observed. The highest anti-parasitic effect of this extract was related to the concentrations (8, 4, and 2 g/l), which led to a 100% reduction of theronts in 6 hours, and also a concentration of 1 g/l, which led to a 90% reduction of theronts. The lowest anti-parasitic effect belonged to the lowest dose used, i.e., 0.5 g/l. Therefore, it can be said that its anti-parasitic effect is suitable against the *Ichthyophthirius multifiliis* parasite in laboratory conditions. Also, in the present study, the toxicity of the alcoholic extract of pomegranate peel was investigated in fish. The results of the investigation of the alcoholic extract of pomegranate peel showed that the concentrations of 2 and 4 g/l of this extract had high toxicity, which led to the death of all fish in 24 and 48 hours, respectively. Concentrations of 1 and 0.5 g/L

did not cause any casualties until the end of 96 hours and were reported as safe doses in this part of the experiment. EC50 was also calculated as 1.41 g/L. Therefore, the dose of 1 g/L is recommended as a clinical treatment due to its non-toxicity and 96% effect on the destruction of theronts, but the doses of 2, 4, and 8 are only suitable for disinfection due to their high toxicity for fish and are not effective for clinical treatment.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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