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**Research Article**

# Acute toxicity of hydroalcohlic extracts of *Heracleum persicum* (Golpar) in zebrafish (*Danio rerio* Hamilton)

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**Abstract**

*Heracleum persicum* (HP) is an annual herbbelonging to Apiaceae (Umbelliferae) family and traditionally cultivated in Iran for its medicinal properties. Preliminary phytochemical analysis of *H. persicum* extract has shown that it possesses antioxidant properties, which may be attributed that to the presence of furanocoumarin compounds. In an experiment involving *Danio rerio* were, six groups of fish were exposed to different concentration of HA-PA ranging from 470 to 500 mg distributed among six groups in triplicates included G1 (control), G2 (1000 mg/kg), G3 (2000 mg/kg), G4 (4000 mg/kg), G5 (8000 mg/kg) and G6 (16000 mg/kg). The probit value for HA-HP and associated concentrations indicated low toxicity in *D. rerio*. The LC50 of HA-HP for *D. rerio* exposed to various concentrations for 24 hrs was found to be 6020.37 mg/kg with lower and upper bounds of 4477.5 and 8117.9, respectively at a 95% confidence limit. The maximum value of buccal movement rate was observed in animals exposed to 16000 mg/kg of HA-HP measuring between 134.0-192.66 N/min. However, increased doses of HA-HP led to respiratory distress and a decrease in oxygen uptake in fish. Moribund fish were observed at 1000 and 2000 mg/kg with signs of darting swimming, circular movement, and settling on the bottom of the aquarium for up to 12 hrs. The exposure of *D. rerio* to increasing concentration of HA-HP also resulted in the Loss of schooling behavior, which could affect their ability to move in coordination with their peers. Based on these findings, it can be concluded that the LD50 of HA-HP for *D. rerio* after 24 hrs is high indicating it can be used safely as an immunostimulant or for other purposes in fish at lower dosages.

**Keywords:** Zebrafish, *Heracleum persicum*, Lethal Dosage, Aquaculture

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## Introduction

Medicinal plants contain a wide range of natural chemical compounds that possess various pharmacological and therapeutic properties. These compounds are often used in nutritional supplements and culinary preparations. However, the safety of these products must be evaluated to ensure they do not pose any harmful effects on human health. Thus, it is important to assess the toxicity of medicinal plants to determine their potential risks and ensure they are used safely (Modarresi Chahardehi *et al.*, 2020).

*Heracleum persicum* known as "Golpar" in Iran is an annual herb from the Apiaceae (Umbelliferae) family with various therapeutic properties (Majidi and Lamardi, 2018). Preliminary phytochemical analysis of *H. persicum* extract has revealed the presence of volatile oils, alkaloids, flavonoids, furanocoumarins, terpenoids, triterpenes and steroids (Sayyah *et al.*, 2005). Traditional Iranian literature mentions *H. persicum* as having anti-fungal, anti-convulsant, anti-microbial, anti-oxidant, anti-inflammatory, analgesic, and immunomodulatory activities (Choi and Hwang, 2004; Küpeli *et al.*, 2006). Studies have shown that *H. persicum* possess antioxidant properties, which can help inhibit the normal functioning of cells by trapping free radicals, the main cause of inflammation (Moreno *et al.*, 2020). Consequently, the furanocoumarins present in *H. persicum* have anti-inflammatory effects by inhibiting free radicals (Hemati *et al.*, 2012). that is due to the presence of furanocoumarin compounds (Souri *et al.*, 2004). is the presence of free radical compounds. The herbal plants contain antioxidant compounds that can be trapped free radicals inhibiting the normal functioning of cells. Accordingly, the antioxidant compounds present in this plant (furanocoumarins) cause anti-inflammatory effects by inhibiting free radicals *(*Hemati *et al*., 2010*).* They documented the Effect of the HA-HP (Golpar) on folliculogenesis in female Wistar rats.

On the other hand, the traditional usage of plants/parts in fish food (such as leaves, seeds, bark and roots) have varying potencies in aquaculture (Tewari and Kaur, 2022). Kakoolaki *et al.* (2016) demonstrated that incorporating *Camellia sinensis* into the diet of *Mugil cephalus* enhances their innate non-specific responses, hematological parameters, and growth performance when exposed to *Photobacterium damselae*. Medicinal plants have various biological functions in fish, including reducing stress, antimicrobial activities, boosting immune function, and promoting growth (Rummun *et al.*, 2017). To increase the net profit of fish farming and reduce the costs associated with fish feed, cost-effective plant-based sources may serve as suitable alternatives for inclusion in formulated feed. The use of these natural resources can be highly beneficial in ensuring the sustainable development of aquaculture in terms of environmental, social, and economic efficiency. (Hambrey, 2017; FAO, 2018). With the expansion of

aquaculture practices, there is an urgent need to find alternative sources of fish feed without increasing operational costs. Considering this issue, the use of non-conventional plant sources in the aquatic feed will be of great importance (Tewari and Kaur, 2022). In addition, these plant resources offer several other benefits such as fish pond fertilization, biological treatment, etc., along with improving the farmer's income through integrated farming (Kumar *et al.*, 2017). Due to the widespread use of *H. persicum* fruits as a medicinal plant and Iranian folkloric claims regarding their analgesic and anti-inflammatory effects, it was deemed necessary to evaluate the lethal dosage (LD50) of the hydroalcoholic extract of *H. persicum* fruits in zebrafish, an animal model. The objective of this research was to apply the data obtained to the field of aquaculture. Based on the afore-mentioned results, the aim of this study was determined the physio-toxicity as well as Median Lethal dose (LD50 value) of *H. persicum* extracts in zebrafish over a 24 hrs period. The extract was tested at five concentrations (1000, 2000, 4000 and 16000 mg/kg).

## Materials and methods

*Animals*

As an animal model, wild-type zebrafish (*D. rerio*) of both sexes, approximately 4 months old, were purchased from a private sector in Tehran, Iran. A total of 108 fish were transferred to the laboratory, where the aquarium was designed and set up. The fish were acclimated in a large glass aquarium pre-filled with aerated water, set at a temperature of 28°C using an automatic thermostat, with a pH range of 7-7.5 and a 14:10 light:dark cycle, respectively, for a period of two weeks (Westerfield, 2000). They were fed twice a day The animals were fed twice a day with artemia in the morning and with a commercial ration in the afternoon at the ratio of 3% of body weight.

*Herbal preparation and extraction*

The fruits of *H. persicum* (HFP) were collected from the suburbs of Shemiran, located in northern Tehran, Iran. Samples were air-dried under natural conditions and powdered using herbarium techniques (Chemicals, 2005). He extraction method was based on the guidelines of the Organization for Economic Co-operation and Development. To prepare the hydroalcoholic extraction of *H. persicum*, 200g of the fruit was powdered and air-dried, then soaked in 1500 mL of a 1:1 EtOH-H2O solution for 48 hours. The combined extract was filtered and evaporated to dryness for 5-6 hours, and then stored in a refrigerator at 4°C until used.

*Experimental design*

The experimental groups were illustrated in Figure 1. After acclimatization, fish with a weight range of 470 to 500 mg were distributed into six groups, each with six fish in triplicate. The experimental design consisted of six groups: G1 (control), G2 (1000 mg/kg), G3 (2000 mg/kg), G4

(4000 mg/kg), G5 (8000 mg/kg), and G6 (16000 mg/kg), as shown in schematic 1.

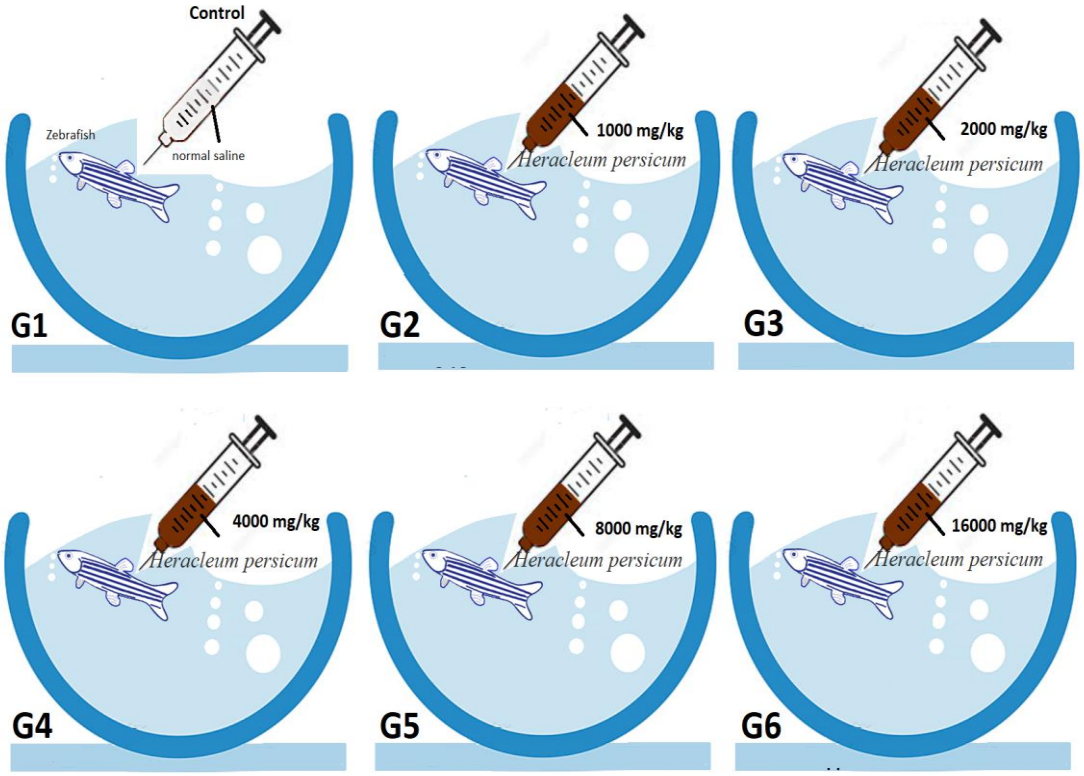


**Figure 1: The schematic feature shows the study design of the experiment with 6 fish each aquarium for ip injection of *Heracleum persicum* (Golpar) extract at different dosage into Zebrafish (*Danio rerio*)**

*HA-HP administration*

HA- HP was administered by dissolving it in water using a water-based approach for medicine administration, at the doses mentioned previously. According to this approach, the medicine was directly dissolved in the water based on the volume of the water (Husnul *et al.*, 2015). Therefore, HA-HP was directly dissolved in one liter of water in a jar, and different concentrations were then prepared according to the study protocol.

*Dose adjustment*

For this trial study, zebrafish with a weight range of 470 to 500 mg were selected. The stock solution of HA-HP was prepared based on the groups specified in the experimental design and calculated in mg/mL. Each zebrafish was administered with a 10 µL volume of the prepared HA-HP working solution at a concentration given in Figure 1, using a 30-gauge syringe (Stewart *et al.*, 2011).

*Injection of HA-HP*

HA-HP was administered intraperitoneally (i.p.) into the abdominal cavity of the fish (Stewart *et al.*, 2011). Briefly, each zebrafish was sedated by soaking it in cold water until

it presented a lack of locomotion and a drop in respiration rate. The fish was then placed on a wet sponge, and the i.p. injection was carried out according to the aforementioned protocol. The treated fish was then placed in a separate jar pre-filled with clean and aerated water until it had fully recovered.

*Median lethal Dosage (LD50 24 h) of plant extract*

Acute toxicity of HA-HP fruit extract samples was tested in the zebrafish model (*D. rerio*) as described by OECD guidelines (OECD, 1992). The zebrafish were exposed to the test substance was set for a period of 24 hours. Mortalities were then counted at each hour until the end of experiment. The median lethal dosage (LD50 24 h) of HA-HP extract that caused 50% of fish mortalities was deployed.

*Respiratory analysis*

The buccal movement rate of fish of each group and replications (3 samples) was calculated as number each minute. The opercular movement rate (OMR) as number(N)/min., which shows the respiratory activity was also measured.

The fish buccal and opercular activity at each minute were totaled twice an hour, during 24 h, in both the control and treated groups. The graphic illustration of the both mean values attained at each dose per time was then designed (Kishore *et al.*, 2022). The video images were made with an inverted microscope (Zeiss, Austria) that was linked to a digital video camera (Canon, Japan) so that the buccal and opercular movement to be seen better (Yaqoob and Schwerte, 2010).

*Behavioral analysis and mortality*

To test the effects of HA-HP on the control and test groups, physiological reactions were observed and recorded. These reactions were classified into three stages: (1) amplification of movement activity, shock, and contractions in the tail axis; (2) circular swimming and loss of attitude; and (3) tetanus, immobility, relocation of the fish to the bottom of the aquarium, and death. Each zebrafish was assessed individually, and it was considered a dead animal when activity of the buccal or operculum and the reaction to mechanical stimulation could no longer be observed (Souza *et al.*, 2016).

*Statistical analysis*

One-way analysis of variance (ANOVA) was used to determine the differences of LD50 among the groups followed by a post hoc multi-comparison, Tukey test. Data are presented as Mean±SE at 0.05 level of probability. GLM-Univariate followed by Tukey test was used to evaluate buccal and opercular movement rate per minute. Probit confidence limit estimation and LD50 calculation were carried out using SPSS, V.26 for Windows.

**Results**

Table 1 presents the acute toxicity values of HA-HP in adult zebrafish during a 24-hour exposure period. No mortality was observed in the control group (G1) during the study.

The mortality of D. rerio in G2 and G3 ranged from 0.0-16.6% and 0.0-22.22%, respectively, indicating an increase in mortality in a concentration-dependent and time-dependent manner. This range gradually increased to values of 0.0 - 22.22%, 16.6 - 55.55%, and 44.4 - 100.0%, respectively, in G4, G5, and G6. Among the treatment groups, the LC50 was observed at a 24-hour exposure concentration of 8000 mg/kg in G5 of D. rerio (Table 1).

**Table 1: Sets of experimental bioassay data percentage mortality in different dosages and time (*n*:6)**

| | | Time (hours) | | | |
|---|---|---|---|---|---|
| | | 1 | 6 | 12 | 24 |
| **G1** | n | 0.0±0 | 0.0±0 | 0.0±0 | 0.0±0 |
| | % | 0.0±0 | 0.0±0 | 0.0±0 | 0.0±0 |
| **G2** | n | 0.0±0 | 0.33±0.57 | 0.66±0.57 | 1.0±0.0 |
| | % | 0±0 | 5.55±9.62 | 11.11±9.62 | 16.6±0.0 |
| **G3** | n | 0.0±0 | 1.0±0.0 | 1.0±1.0 | 1.33±0.57 |
| | % | 0.0±0 | 16.6±0.0 | 16.6±16.6 | 22.22±9.62 |
| **G4** | n | 1.0±0.0 | 1.66±0.57 | 2.0±0 | 2.33±0.57 |
| | % | 16.6±0.0 | 27.27±9.62 | 33.3±0 | 38.88±9.62 |
| **G5** | n | 1±1.0 | 2.33±0.57 | 2.66±0.57 | 3.33±1.15 |
| | % | 16.6±16.6 | 38.88±9.62 | 44.44±9.62 | 55.55±19.24 |
| **G6** | n | 2.66±0.57 | 4.0±0 | 5.33±0.57 | 6.0±0 |
| | % | 44.44±9.62 | 66.66 | 88.88±9.62 | 100±0 |

G1: Control, G2: 1000 mg/kg, G3: 2000 mg/kg, G4: 4000 mg/kg, G5: 8000 mg/kg, G6: 16000 mg/kg, n: number of fish, %: percent of mortality of each group

Figure 2 depicts the probit value for HA-HP and its associated concentrations. Accordingly, the LC50 of HA-HP for D. rerio exposed to various concentrations for 24 hours was 6020.37 mg/kg, with lower and upper bounds of 4477.5 and 8117.9, respectively, at a 95% confidence limit for concentration. This probit model can account for 80.6% of the changes that may be observed in the future (Fig. 2).

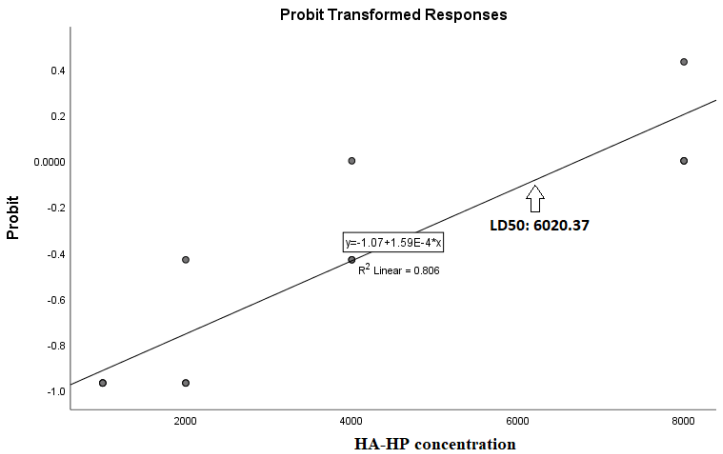


**Figure 2: Regression scatter plot with trend line between the of concentration and probit value of HA-HP (mg/kg adult *Danio rerio*) at 24 hrs exposure time (p<0.05)**

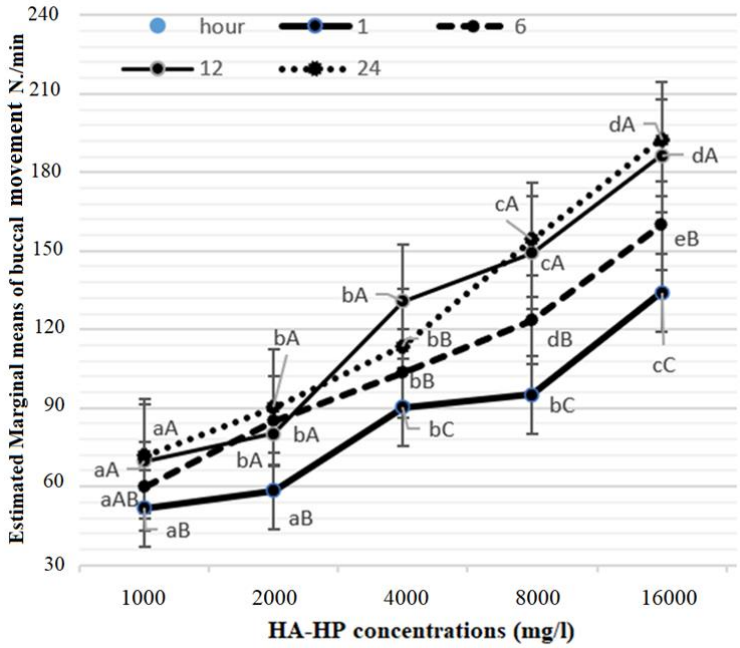


**Figure 3: Changes in operculum rate (Number/min) in the adult *Danio rerio* exposed to different concentrations of *Heracleum persicum* (Golpar) during 24 hrs exposure**

Figure 3 depicts the estimated marginal mean of buccal movement rate per minute. According to the Univariate GLM test, the partial Eta squared value showed that the model was fitted to the data at 90.7%. The control group showed a buccal movement rate of 40 N/min, which increased in a time-dependent manner. The minimum value of buccal movement ranged from 51.6 (with no significant difference compared to the value of 2000 mg/kg of HA-HP) to 71.66 N/min from 1 to 24 hours of exposure to 1000 mg/kg HA-HP in zebrafish. The maximum value was observed in animals exposed to 16000 mg/kg of HA-HP, with a range of 134.0-192.66 N/min. The buccal movement of animals exposed to 8000 and 16000 mg/L of HA-HP was 149.3 and 186.3 N/min after 12 hours, with no significant difference ($p$>0.05) compared to animals after 24 hours in the same groups.

Figure 4 shows the estimated marginal mean of the number of opercular movements (OM) per minute. According to the partial Eta squared value, the two-way ANOVA model was fitted to the data at 98.2%. The control group showed an OM rate of 35 N/min, which increased in a time-dependent manner, particularly with increasing HA-HP concentration. The minimum value of OM was observed on day 1, ranging from 51.6 to 71.6 N/min, while the zebrafish were exposed to different concentrations of HA-HP. For the time of 6 hours, this value started with 60.0 N/min at 1000 mg/kg and ended with 159.6 N/min at 16000 mg/kg. The differences in the number of OM per minute were not significant ($p$>0.05) after 6 hours, either for 1000 or 2000 mg/kg.

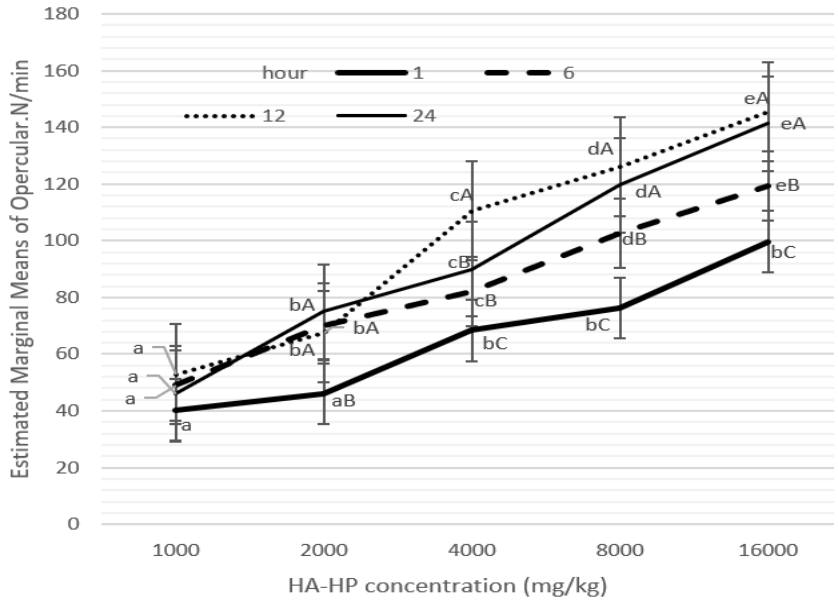


**Figure 4: Changes in operculum rate (Number/min) in the adult *Danio rerio* exposed to different concentrations of *Heracleum persicum* (Golpar) during 24 h**

**Table 2: Clinical sign of *Danio rerio* reaction after exposure to *Heracleum persicum* (Golpar) at different concentrations during 6- hrs interval samplings**

| C (mg/kg) | Hours | Total number | Stage1 | Stage 2 | Stage 3 | Total | % |
|---|---|---|---|---|---|---|---|
| 0 | 1-24 | 18 | 0/18 | 0/18 | 0/18 | 0/18 | 0 |
| 1000 | 1 | 18 | 1/18 | 0/18 | 0/18 | 1/18 | 5.55 |
| | 6 | 18 | 1/18 | 1/18 | 1/18 | 3/18 | 16.66 |
| | 12 | 17 | 2/17 | 1/17 | 2/17 | 5/17 | 29.41 |
| | 24 | 15 | 2/15 | 1/15 | 3/15 | 6/15 | 40.0 |
| 2000 | 1 | 18 | 1/18 | 0/18 | 0/18 | 1/18 | 5.55 |
| | 6 | 18 | 1/18 | 1/18 | 1/18 | 3/18 | 16.66 |
| | 12 | 17 | 2/17 | 1/17 | 2/17 | 5/17 | 29.41 |
| | 24 | 15 | 3/15 | 2/15 | 3/15 | 8/15 | 53.33 |
| 4000 | 1 | 18 | 1/18 | 1/18 | 3/18 | 5/18 | 27.77 |
| | 6 | 15 | 2/15 | 2/15 | 5/15 | 10/15 | 66.66 |
| | 12 | 10 | 2/10 | 1/10 | 2/10 | 5/10 | 50.0 |
| | 24 | 8 | 1/8 | 2/8 | 3/8 | 6/8 | 75.0 |
| 8000 | 1 | 18 | 1/18 | 2/18 | 3/18 | 6/18 | 33.33 |
| | 6 | 15 | 3/15 | 3/15 | 4/15 | 10/15 | 66.66 |
| | 12 | 11 | 1/11 | 2/11 | 2/11 | 5/11 | 45.45 |
| | 24 | 9 | 2/11 | 2/11 | 5/11 | 9/9 | 100.0 |

**Table 2 continued:**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 16000 | 1 | 18 | 1/18 | 2/18 | 2/18 | 5/18 | 27.77 |
| | 6 | 16 | 2/16 | 2/16 | 4/16 | 8/16 | 50.0 |
| | 12 | 12 | 1/12 | 1/12 | 5/12 | 7/12 | 58.33 |
| | 24 | 5 | 0 | 0 | 5/5 | 5/5 | 100.0 |

C: Concentration,

The physiological reactions of *D. rerio* to the HA-HP were converted to qualitative statistics and given in Table 2. Accordingly, the clinical signs observed in response to HA-HP was gradually increased so that the maximum percent of clinical cases was 40.0, 53.3 75.0, 100.0 and 100.0%, respectively in groups;1000, 2000, 4000, 8000 and 16000 mg/kg. The moribund fish of G2 was 50% of cases including the fish usually showed darting movement, stressfulness and tail muscle contraction. This value reached 62.5% after 24 hrs in G3, in which the concentration of HA-HP increased two-times more compared with G1. In G4 and G5, approx. 72.2% of cases were dead after 24 hrs and survived fish were suffering from difficulties in movement or respiration. The fish were completely died in G6 24 hrs of exposure. Exposed *D. rerio* exhibited Lack of adequate balance with the increased HA-HP concentrations (1000-4000 mg/kg) and exposure time (6-12 hrs). In control fish, they normally moved and swam all spaces of water column. They eventually moveless at the bottom and dead at higher concentrations (8000-16000 mg/kg) of the HA-HP and exposure time.

## Discussion

The present study aimed to analyze the toxicity of *H. persicum* (Golpar) in adult *D. rerio* exposed to different concentrations. Prior to the experiment, the cytotoxicity of HA-HP was tested on artemia larvae and Swiss albino mice, and the results showed an appropriate margin of safety with an LD50 above 2000 mg/kg body weight at an exposure time of 96 hours (Chacha and Mbugi, 2019). Another research showed that the toxicity evaluation with 100 µg⁄mL of the chloroform fraction of HA-HP could induce the highest (97%) mortality rate in *Artemia salina* (Mofasseri *et al.*, 2017) but no description about the dosage per animal weight explained. This finding was not in line with this study showed LD50 of HA-HP for 24 hrs was 6020.37 mg/kg in *D. rerio*.

The buccal and opercular activity rates of 1000-2000 mg/kg at 6 hrs after injection was no significant different with those of 12 and 24 hrs in this study. The rate of buccal movement in the exposed fish decreased with time increased during 96 hrs (Kishore *et al.*, 2022) against this study showed the buccal movement rate increased in time dependent manner. This difference might be due to the former study showed a decrease during 96 hrs and our research

was performed in 24 hrs. They showed that the buccal movement rate was decreased in herbal concentration dependent manner.

Since buccal activity in fish is associated with respiratory activity (Soni and Verma, 2018), the increased dose of HA-HP in zebrafish led to respiratory distress and a gradual decrease in oxygen uptake in fish. The increased opercular activity observed in the treated fish following raised HA-HP could be due to the constant toxic conditions activating sensory inducement to increase opercular movement for appropriate respiratory function of the gills to manage hypoxia caused by the drop in oxygen uptake through the gills (Baruah and Das, 2002). On the other hand, the gradual decreases in oxygen consumption rate in the fish strongly indicates the onset of acute hypoxia under toxicant-induced stress (Vutukuru *et al.*, 2005). A typical resting respiratory rate for adult zebrafish is about 160 number.min$^{-1}$; while hypoxia may raise respiratory rate to above 300 number. min-$^{1}$ (Jonz and Nurse, 2006). LC50 at 24 hrs of HA-HP exposure of zebrafish in the present study was 6020.37, which was greater than the LC50 of tests conducted with Brine shrimp cytotoxicity assay of extracts for H-HP in disc method (966.44 µg/mL) at 24 hrs (Moshafi *et al.*, 2010). These results suggest that *H. persicum* may act as an immunostimulant, and a lower amount of it can be safely used. In a study evaluating the diet of common carp (*Cyprinus carpio*) enriched with *H. persicum* as a phytoimmunostimulant at three different inclusion levels of 2500, 5000, and 10000 mg/kg, the 5000 mg/kg diet was found to be more effective in enhancing total Ig, lysozyme, and complement activity. (Hoseinifar *et al.*, 2016). LD50 of *H. persicum* seed acetone extract on mice was 1103 (988.2–1245.9) mg/kg with ED50 of 4.15 and 5.35 mg/kg against seizures.

Based on the results of Table 2, The Moribund fish were suffering from HA-HP usually at 1000 and 2000 mg/kg with signs of darting swimming, circular movement, settlement on the bottom of aquarium until 12 hrs. Other studies have exhibited that environmental relevant toxicant in fish can lead lethargy, loss of appetite, anorexia, muscle contraction, stressed swimming, body balance loss, skin darkening, decreased or increased respiratory rate, and finally death (Sahu and Kumar, 2021). Schooling behavior in fish was supposedly initiated on chemical compound and locomotor responses (Pavlov and Kasumyan, 2000), as such the loss of schooling behavior in exposed *D. rerio* in this study that increased concentrations of HA-HP could induce responses in fish could change their ability for coordinated movement within a school. Dislocated ability for spatial recognition such as spatial memory and learning capability particularly in schooling with dreadful implications for fish fitness and survival in the wild have been exhibited following exposure to toxicants (Ward *et al.*, 2008; Jacquin *et al.*, 2020).

Based on the afore-mentioned discussion, it is concluded that LD50 of

HA-HP for *D. rerio* at 24 hrs is great and it can be used as immunostimulant or other usage in fish in a safe margin at low dosage.

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