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Biopesticidal Efficacy and Safety of Azadirachtin: Broad-Spectrum Effects on Ectoparasites Infesting Goldfish, *Carassius auratus* (Linn. 1758)

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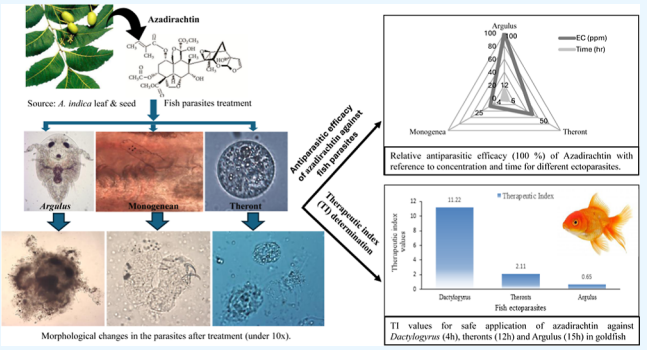
ABSTRACT: Ectoparasites are a serious concern for the ornamental fish industry, causing severe pathological and behavioral changes that diminish the aesthetic value of fish and adversely affect trade. Recently, phytotherapy, application of plant extracts or their active biomolecules, has gained attention for the control and treatment of various diseases of humans and animals, including fish. The present study evaluated the broad-spectrum antiparasitic activity of azadirachtin, a neem-based bioactive molecule, under *in vitro* condition against adult protozoan (*Ichthyophthirius multifiliis*) and adult metazoans ectoparasites, including *Dactylogyrus* sp. and *Argulus* sp., as well as juvenile stages and eggs of the latter, for its prevalence in infesting goldfish (*Carassius auratus*). Azadirachtin was tested at concentrations of 9.52−47.61 mg L^{-1} against *I. multifiliis* theronts; 1−30 mg L^{-1} for *Dactylogyrus* sp.; and 25−125 mg L^{-1} for *Argulus* sp. The median effective concentration (EC_{50}), antiparasitic efficacy (AE), relative antiparasitic efficacy, and therapeutic index (TI) were estimated for azadirachtin. EC_{50} of azadirachtin against different parasites was estimated to be in the range of 6.08 to 61.29 mg L^{-1}. Relative antiparasitic efficacies were found to be 25 mg L^{-1} for 4 h, 50 mg L^{-1} for 6 h, and 100 mg L^{-1} for 12 h against *Dactylogyrus* sp., *I. multifiliis* theronts, and *Argulus* sp., respectively. *Argulus* egg hatching was inhibited above 100 mg L^{-1}, with eggs at 125−175 mg L^{-1} becoming unclear by day 14. The 15 h median lethal concentration (LC_{50}) of azadirachtin (EC = 21.5%) against *C. auratus* was 20.48 mg L^{-1}. The TI indicates a safe dose in *C. auratus* against *Dactylogyrus* sp. (11.22 for 4 h) and *I. multifiliis* theronts (2.11 for 12 h) while showing a critical value for *Argulus* (0.65 for 15 h). The present findings provide evidence for the broad-spectrum antiparasitic activity of azadirachtin against protozoan and metazoan ectoparasites, suggesting its potential as an agent for controlling a wide range of ectoparasites in ornamental fishes.



1. INTRODUCTION

Goldfish (*Carassius auratus*) is the most popular aquarium fish, known for its diverse variety, coloration, and body shape. It is used as a model fish in various fields of research like auditory research, toxicity study, teleostean visual system, neuropharmacology, etc.[1−3] However, the rising global demand of aquarium fish has encouraged farmers for intensive culture in a confined environment and impetuous handling. As ornamental fishes are forced to remain under highly crowded and artificial conditions, they are susceptible to a wide range of bacterial, viral, and parasitic diseases.[4,5] Among the wide array of parasites, infestation with ectoparasites is ubiquitous in the aquatic environment and is calamitous on aquatic animals cultured in ponds and aquaria.[6] Ectoparasites of teleost range from unicellular protozoan (ciliophores and flagellates) to metazoan including platyhelminthes (monogenean), crustaceans (copepods and branchiurans), and leeches (hirudenian). The most prevalent ectoparasites which are of concern in freshwater aquarium fishes are *Ichthyophthirius* sp., *Trichodina* sp., *Ichthyobodo* sp., monogenean (*Dactylogyrus* and *Gyrodactylus*), *Lernaea* sp., and *Argulus* sp.[7] These reside on the body of the host including the skin, gills, and fins and may cause pathological alterations such as hemorrhages, anorexia, irritability, ataxia, anemia, skin necrosis and erosion, dermatitis, and detrimental immune reactions (hypersensitivity, anaphylaxis, etc.) under

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moderate and heavy infestations. With alteration in culture conditions, it becomes a potential source of subsequent secondary infections aggravating the total damage.[8−11] Moreover, detailed information on economic loss and impact on trade caused by these ectoparasites is merely available. Therefore, studies of the impact of these ectoparasites and their potential therapies are urgently needed.

Since long, chemicals are being used for treating and controlling of ectoparasites in aquaculture, such as salt (sodium chloride), potassium permanganate, formaldehyde, formalin, trichlorfon, emamectin benzoate, powdered quicklime, copper sulfate, etc.[12−15] Moreover, traditional parasiticides such as praziquantel,[16] trichlorfon,[17] and mebendazole[18] have been used for controlling monogenean infestations. Recently, a wide array of therapeutic approaches are being critically appraised to control *Argulus* sp.[11] The use of chemicals or synthetic drugs for treating ectoparasites has various side effects, such as animal health hazard,[19] development of resistance in pathogen,[20] and environmental damages,[21] which calls for an alternative safe therapy. Herbal remedies are prevalent in developing countries for human therapy, but little research has been done about their use in the treatment of fish diseases.[22,23]

Phytotherapy is an excellent alternative multicomponent therapy, targeting a wide variety of pathogens, consisting of a cocktail of components either acting positively (synergistic) or negatively (antagonistic) to each other, thereby producing a therapeutic effect.[24] Compounds from garlic have been reported to be effective against *Gyrodactylus turnbulli*,[25] Trichodinids,[26] and theronts and tomont stages of *Ichthyophthirius multifiliis*.[27] Azadirachtin, a secondary metabolite present in neem (*Azadirachta indica*) plant, has been found to be effective against *Argulus* sp.[28] Piperine from *Piper longam* and methanol extracts of *Piper guineense* have been found to be effective against *Argulus* sp.[29] and monogenean,[30] respectively. In most cases, either extract or single bioactive compound of herbs is being evaluated for its antiparasitic efficacy (AE) against a parasite or its larval stages. The spectrum of the antiparasitic effect is merely tested, and very scarce literature is available on herbal bioactive molecules against groups of fish parasites. Azadirachtin is a highly oxygenated triterpenoid having antimicrobial, antifungal, and pesticide properties.[31] In light of these aspects, the present study was performed to evaluate the spectrum of antiparasitic effects of azadirachtin and its relative antiparasitic efficacy (RAE) against common ectoparasites of *C. auratus*.

2. MATERIALS AND METHODS

2.1. Experimental Fish and Sampling for Isolation of Different Ectoparasites. Parasite- infested goldfish (*C. auratus*) (4.75 ± 0.25 g) were obtained from local vendors in Mumbai, Maharashtra, India. On arrival of fish, sampling was carried out to segregate fish infected with different parasites. The *Argulus* infestation was identified visually by the presence of *Argulus* attached on the body surface of the fish (Figure S1), the ciliate *I. multifiliis* infestations were identified visually by the presence of white spots on the fish body followed by observing a mucous smear under a microscope for confirmation (Figure S2), while monogenean (*Dactylogyrus* sp.) infestation in fish was identified by examining small excised gill samples from ten fish and observed under a microscope (40×) (Figure S3). Fish were then segregated and maintained at 23 ± 2 °C under aerated conditions in 150 L glass tanks according to the presence and prevalence of different parasites until the parasites were subjected to the *in vitro* test. Presence of more than 10 *Argulus* on the body, 50−60 *Dactylogyrus* per fish, and numerous white spots on the body of fish were considered as infested fish.

2.2. Artificial Infestation of Parasites in Goldfish. To ensure a consistent supply of stage-specific ectoparasites, *Argulus*-infested fish (identified during sampling) were used to artificially infect healthy fish through two methods: (1) cohabitation method:[32] heavily and moderately *Argulus*-infested fish were housed with healthy fish at a 1:5 ratio in shared aquaria. Infested fish were marked by a small cut on the caudal fin to monitor the spread of infestation. Daily observations were made for one week to track parasite proliferation and spread. (2) Substrate-based modified method:[28] substrates such as stone slabs, plastic aeration pipes, *Hydrilla* macrophytes, bamboo sticks, and stones were placed in a tank with *Argulus*-infested fish. After spawning, the egg-laden substrates were transferred to separate tanks containing healthy goldfish for infestation. All the tanks used for artificial infestation of *Argulus* were labeled.

Additionally, to obtain sufficient theronts for the experiment, an artificial infestation of *I. multifiliis* in goldfish was performed using the cohabitation method. Healthy goldfish were placed with infested fish in the same tank, and daily observations tracked parasite proliferation and spread. In the case of fish mortality, skin scrapings containing parasites were collected and released back into the tank, and healthy fish were added to continue the infestation cycle.

2.3. Cleaning and Siphoning. Tanks were cleaned manually prior to any experiment. Before introducing new fish, tanks were filled with water 24 h in advance with continuous aeration. Daily siphoning was performed in tanks with healthy fish to remove settled feed pellets and fecal matter, replacing the siphoned water with an equal volume of borewell water at the same temperature.

For tanks with *Argulus*-infested fish, siphoning was done once every 10 days. The siphoned water, containing feed and fecal matter, was transferred to a tub and replaced with an equal volume of borewell water. To capture any*Argulus* removed during siphoning, 2−3 healthy goldfish were placed in the tub containing the siphoned water and later returned to the tank with infested fish. If there was a decrease in the number of *Argulus* after siphoning, then the tanks were left undisturbed until the infestation levels were restored. The physicochemical parameters of the water were as follows: temperature 23.5−31.2 °C, pH 7.3−8.1, dissolved oxygen 6.4−7.4 mg L^{-1}, and alkalinity 182−208 mg L^{-1}.

2.4. Preparation of Stock of Diluent Control and Working Test Solution. Technical grade azadirachtin powder (EC 21.5%) (SOM Phytopharma, Hyderabad, India) was used for the preparation of stock and test solution. The stock solution of concentration 1 g L^{-1} was prepared by dissolving 1 g of azadirachtin powder (EC 21.5%) in 5 mL of methanol (Vittal Mallya Scientific Research Foundation, Bengaluru, India) and finally making the total volume to 215 mL by addition of distilled water. The working test solutions of different concentrations for experimentation against different ectoparasites were made by using the stock solution. The stock and working test solutions were freshly prepared on the day the tests were performed.

2.5. Experimental Design for *In Vitro* Antiparasitic Test of Azadirachtin against Each Parasite. A pilot range-finding study was conducted using varying concentrations of azadirachtin to determine the optimal concentrations and treatment duration for the *in vitro* assay against specific ectoparasites (see Table S1). The methodology for the final *in vitro* bioassay is detailed below.

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2.5.1. Experiment 1a: Collection of Argulus sp. and Estimation of In Vitro AE of Azadirachtin against It. *In vitro* AE of azadirachtin against *Argulus* sp. was evaluated using the method followed in our lab, as described previously.[28] *Argulus* were taken from infested fish by shaking the fish gently for 2 min in a Petri plate containing 100 mL of water, and parasites were gently picked from the host using fine plastic forceps taking care not to damage the parasite. The *in vitro* tests were performed in triplicates, and the parasites were exposed for 15 h to 5 different concentrations of azadirachtin, namely, 25, 50, 75, 100, and 125 mg L^{-1}; a positive control containing 2.32% methanol without azadirachtin; and a negative control containing filtered aquarium water was also maintained. From the collected *Argulus*, 18 active swimming adult parasites of the same size group were randomly selected and transferred into each Petri plate containing 50 mL of working test solution using fine plastic forceps. At every 30 min, the parasites were observed for mortality. Parasites were considered as dead by the absence of motility after observation for 5 min and an additional observation by nudging with a forcep for 5 min.

2.5.2. Experiment 1b: Collection of Argulus Eggs and Estimation of In Vitro AE of Azadirachtin against It. *Argulus* eggs were collected from the tank containing artificially infested goldfish with *Argulus*. The confirmation of the eggs belonging to the same stages was done by observing them under a microscope for the presence of eye spots and crudely by noticing their color. Daily observations of eggs in the tanks facilitated the selection of eggs at the same developmental stage. Post observation, the clutch was divided in such a way that each subsection consisted of 30 eggs each.

Thirty eggs were exposed to 50 mL of solution containing different concentrations of azadirachtin, namely, 0 (control), 25, 50, 75, 100, 125, 150, and 175 mg L^{-1} in Petri plates. The Petri plates were maintained in natural photoperiod (12:12 h) of light and darkness for the development and hatching of eggs. The temperature during the experimental period was 29.5−31.5 °C. The eggs were observed at every 12 h until 100% hatching occurred in the control groups. The solutions in the treatment and control groups were refilled regularly. All tests were performed in triplicates along with a diluent control with 2.5% methanol but no azadirachtin for comparison. The developmental stages of eggs were observed under the microscope by placing the eggs on a cavity slide containing the solution of the Petri plate. Also, care was taken not to damage the eggs.

2.5.3. Experiment 2: Collection of I. multifiliis Theronts and In Vitro Efficacy of Azadirachtin against It. The *I. multifiliis* theronts were collected following the method of Clayton and Price.[33] The heavily infested goldfish were placed in a 100 mL capacity Petri plate containing filtered aquarium water for 30 min. Mature parasites were freely dislodged from the fish due to body movement. Actively moving parasites were transferred to a fresh Petri plate containing filtered tank water with the help of a Pasteur pipet. These parasites were then incubated at 23 ± 0.50 °C for 20−24 h for obtaining the theront stage.

The concentration of theronts was estimated using the method of Straus and Meinelt[34] by placing 10 droplets of 2 μL each on a glass slide and observing the organism under a microscope (Carl Zeiss, Axioskop2, Germany). The mean number of theronts in each droplet was extrapolated to estimate the final concentration of a minimum of 300 theronts. The theronts were placed into 96-well microtiter plates at a final concentration of 300 theronts per well with 200 μL of solutions of different azadirachtin concentrations (rounded/actual values) of 10 (9.52), 20 (19.05), 30 (28.57), 40 (38.09), and 50 (47.61) mg L^{-1}. The positive and negative control groups were also retained with 2.32% methanol without azadirachtin and filtered aquarium tank water, respectively, under the same conditions as the test groups. All treatment groups were kept in triplicate, and mortality in each well was assessed by the microscopic examination; the theront cells with absence of motility and abnormal morphology were considered dead. Post exposure, the theronts were continuously observed for its mortality until all parasites had died in the lowest concentration.

2.5.4. Experiment 3: Collection of Monogenean (Dactylogyrus sp.) Parasites and In Vitro AE of Azadirachtin against It. *Dactylogyrus* sp. were collected from infested goldfish as per Fridman et al.[35] by excising a small portion of the gill and observing it under a microscope (10× magnification) (Carl Zeiss, Axioskop2, Germany) with a minimum of 10 parasites in the examined field. An average of 35−40 parasites were transferred into a well of 96-well microtiter plates containing 200 μL of filtered aquarium water and observed under a dissecting microscope to ensure that the transfer was successful. From here, 30 actively motile parasites were transferred into a well containing 200 μL of test solution with seven different concentrations of azadirachtin 1, 5, 10, 15, 20, 25, and 30 mg L^{-1} in triplicates for estimating *in vitro* AE. Mortalities were noted when parasites appeared with abnormal morphology and a lack of motility even after providing a gentle current of water using a fine needle. A positive control containing 2.32% methanol without azadirachtin and a negative control containing filtered aquarium water were retained along with the treatment groups.

Finally, *in vitro* AE of azadirachtin of each treatment against each parasite was calculated using the following equation[28]

$$\text{AE}\ (\%) = \frac{[B - T]}{B} \times 100\%$$

where AE = antiparasitic efficacy of azadirachtin against *Argulus*, *Dactylogyrus,* and *I. multifiliis*, respectively, B is the mean number of surviving parasites in the positive control, and T is the mean number of surviving parasites in treatment.

The RAE of azadirachtin (EC 21.5%) was considered to be 100% when it remained consistent from a given time point onward, in relation to the lowest concentration of the test compound causing total mortality of the parasites being investigated.

$$\text{RAE} \propto \{C, T\} \text{ when AE is } 100\%$$

where RAE is the relative antiparasitic efficacy of azadirachtin and C is the lowest concentration of azadirachtin at time T when AE is recorded as 100%.

2.6. Acute Toxicity Test of Azadirachtin for *C. auratus* and Estimation of Therapeutic Index. The median lethal concentration (LC_{50}) of azadirachtin (EC 21.5%) solution for *C. auratus* was determined for the estimation of the therapeutic index (TI). A short-term acute toxicity test was conducted according to the standard method.[36] Following an initial range finding study (see Table S1), fish were exposed to 0, 10, 20, 30, 40, and 50 mg L^{-1} of azadirachtin for 24 h, with appropriate controls. All the tests were performed as a static bioassay test in triplicate keeping six fish (mean weight: 3.25 ± 0.25 g) in each tank. No feeding was done during the experiment, and percentage mortality was recorded at 3, 6, 9, 12, 15, and 24 h. Dead fish were removed from the tank immediately. Death was assumed when the fish was immobile and showed no response when touched with a glass rod. Further, the LC_{50} of azadirachtin

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Table 1. Antiparasitic Efficacy (AE %) of Various Concentrations of Azadirachtin (EC 21.5%) against (A) *Argulus* sp., (B) *Dactylogyrus* sp., and (C) *I. multifiliis* Theront at Different Time Periods

A. mean number of surviving *Argulus* sp					AE (%) = (B − T)/B × 100		
azadirachtin (mg L^{-1})	stock	9 h	12 h	15 h	9 h	12 h	15 h
0	18	18 ± 0	18 ± 0	18 ± 0			
25	18	10 ± 2.0	9 ± 1.63	7 ± 1.5	44.44 ± 1.5	50 ± 1.0	61.11 ± 1.5
50	18	8 ± 1.73	6 ± 2.0	3 ± 1.0	55.56 ± 0.75	66.67 ± 1.50	83.33 ± 2.0
75	18	8 ± 1.0	4 ± 1.0	0	55.56 ± 1.5	77.78 ± 1.25	100 ± 0
100	18	6 ± 2.0	0	0	66.67 ± 2.0	100 ± 0	100 ± 0
125	18	3 ± 1.0	0	0	83.33 ± 1.0	100 ± 0	100 ± 0
B. mean number of surviving *Dactylogyrus* sp.					AE (%) = (B − T)/B × 100		
azadirachtin (mg L^{-1})	stock	2 h	3 h	4 h	2 h	3 h	4 h
0	30	30 ± 0	30 ± 0	28 ± 0.5	0	0	0
1	30	30 ± 0	27 ± 0	16 ± 0.66	0	10 ± 0.75	42.85 ± 1.0
5	30	25 ± 0.75	24 ± 1.5	13 ± 0.5	16.67 ± 0.5	20 ± 1.0	53.57 ± 1.33
10	30	26 ± 1.0	21 ± 1.66	9 ± 1.5	13.33 ± 0.66	30 ± 2.0	67.85 ± 1.0
15	30	23 ± 1.0	18 ± 0.50	7 ± 0.66	23.33 ± 1.75	40 ± 2.0	75 ± 2.0
20	30	18 ± 1.5	7 ± 1.5	6 ± 1.5	40 ± 1.0	76.67 ± 1.33	78.57 ± 0.66
25	30	15 ± 2.0	5 ± 1.0	0	50 ± 1.50	83.33 ± 0.66	100 ± 0
30	30	3 ± 1.0	0	0	90 ± 2.0	100 ± 0	100 ± 0
C. mean number of surviving *I. multifiliis* theronts					AE (%) = (B − T)/B × 100		
azadirachtin (mg L^{-1})	stock	3 h	6 h	12 h	3 h	6 h	12 h
0	300	300 ± 0	300 ± 0	300 ± 0	0	0	0
10	300	284 ± 10.33	265 ± 6.67	236 ± 6.0	5.33 ± 0.5	11.67 ± 0.75	21.33 ± 0.5
20	300	248 ± 6.67	206 ± 10.0	0	17.33 ± 1.0	31.33 ± 1.0	100 ± 0
30	300	235 ± 10.33	195 ± 3.67	0	21.67 ± 0.75	35 ± 1.33	100 ± 0
40	300	214 ± 7.66	125 ± 6.33	0	28.67 ± 1.0	58.33 ± 1.0	100 ± 0
50	300	118 ± 6.33	0	0	60.67 ± 1.33	100 ± 0	100 ± 0

for *C. auratus* at various time intervals were analyzed by the probit test. The TI was calculated as the ratio of median lethal concentration (LC_{50}) of fish to median effective concentration (EC_{50})[37] of azadirachtin against each parasite, measured at 4 h for *Dactylogyrus*, 12 h for *I. multifiliis*, and 15 h for *Argulus*, respectively.

2.7. Statistical Analysis. The data were statistically analyzed by statistical package SPSS version 16, and acute toxicity test for medial lethal concentration (LC_{50}) was estimated at the 95% confidence interval by probit analysis.[38] The graphs were prepared using Microsoft Excel 2010.

3. RESULTS

In the present study, we screened goldfish (*C. auratus*) for the presence of common parasites in general. Further, the study evaluated *in vitro* AE of azadirachtin against the most common ectoparasites *Argulus*, *Dactylogyrus* sp. (monogenean) and *I. multifiliis* (ciliate) collected from infested goldfish.

3.1. Prevalence of Different Ectoparasites on *C. auratus*. We examined a total of 150 goldfish, in which 59.33% of fish were infested with ectoparasites (single or multiple infestation). The recorded prevalence of these parasites in the present study was as follows: *Dactylogyrus* sp. (41.33%), *Argulus* (35.33%), and *I. multifiliis* (28.67%). Most preferable sites of attachment were found to be fins, gills, and caudal regions for these ectoparasites. Most of the *Argulus* were found to be attached to the caudal and dorsal fins, and belly regions of the host and large-sized parasite were prominently observed at the caudal peduncle. However, *Dactylogyrus* sp. was anchored on the gills. The ciliate parasite *I. multifiliis* was found to be spread on the body surface preferably in dorsal regions and caudal fins and appeared as white salt granules.

3.2. Artificial Infestation of Goldfish with Common Ectoparasites. *3.2.1. Artificial Infestation of Goldfish with Argulus sp.* Prominent infestation of*Argulus*was observed on goldfish from the third week after the setup of artificial infestation challenge. Each fish was infested on an average with 40−50 juvenile parasites, showing signs such as hemorrhages on fins, slow body movement, reduced feeding, and mortality in the case of heavy infestation (Figure S1). The intensity of *Argulus* on randomly selected ten fish after artificial challenge test is given in Table S2.

During the experiment, the substrates used in the aquaria were examined for the presence of *Argulus* egg clutches. The results showed that aquarium glass surfaces and stone slabs were the most preferred substrates for egg deposition, followed by sticks, stones, aeration pipes, and *Hydrilla*. Substrate preference was inferred from the number of egg clutches and the eggs per clutch relative to substrate size (Figure S4).

3.2.2. Artificial Infestation of Goldfish with I. multifiliis. Healthy goldfish cohabiting with *I. multifiliis*-infested goldfish developed visible infestations within one week of the artificial challenge. Numerous white spots resembling salt granules appeared across the fish's body, excluding the belly region (Figure S2). Key pathological signs include reduced feeding, surfacing, sluggish movement, and mortality. The parasites were predominantly found on the caudal fin, dorsal fin, head, and lateral body surfaces.

Dead fish were removed daily from the tanks and replaced with naive goldfish to sustain the infestation. However, due to high mortality rates and the parasite's intolerance to elevated temperatures, bulk infestations of *I. multifiliis* could not be maintained (Figure S2).

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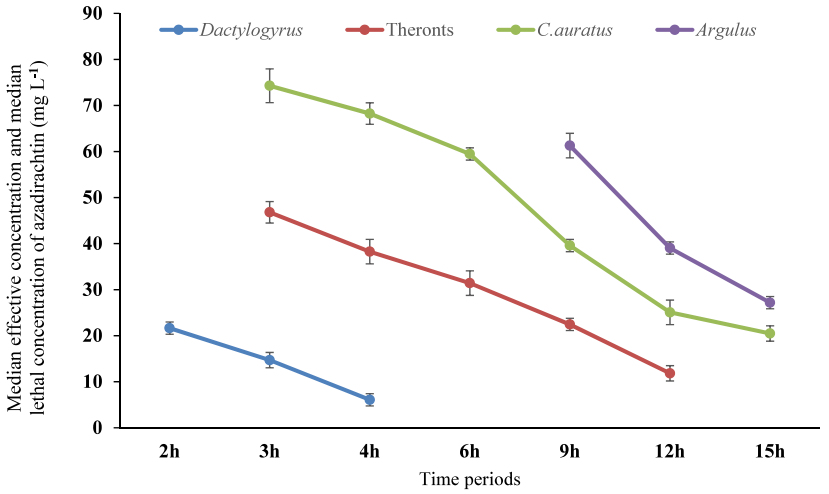

Figure 1. Median effective concentration (EC_{50}) in mg L^{-1} of azadirachtin against three ectoparasites (*I. multifiliis* theront, monogenean *Dactylogyrus* sp., and *Argulus* sp.) and median lethal concentration (LC_{50}) in mg L^{-1} of azadirachtin for host *C. auratus* at various time periods.

3.3. *In Vitro* AE of Azadirachtin against Ectoparasites of *C. auratus*. *3.3.1. AE against Adult Ectoparasites.* *In vitro* antiparasitic efficacy (AE %) of various concentration of azadirachtin against examined adult ectoparasites is presented in Table 1.

The complete elimination of *Argulus*, or 100% AE of azadirachtin against adult *Argulus* sp., was observed at 75, 100, and 125 mg L^{-1} after 15, 12, and 12 h, respectively (Table 1A), as was also observed for juveniles (Table S3). Similarly, 100% AE of azadirachtin against *Dactylogyrus* sp. was observed for 25 and 30 mg L^{-1} at 4 and 3 h, respectively (Table 1B). The concentrations of 50, 75, and 100 mg L^{-1} of azadirachtin solution showed 100% AE against *I. multifiliis* theronts at 12 h and 125 mg L^{-1} at 6 h (Table 1C). The median effective concentration (EC_{50}) of azadirachtin (EC 21.5%) against different ectoparasites was as follows: *Dactylogyrus* sp. (EC_{50}-2 h: 21.65, EC_{50}-3 h: 14.70 and EC_{50}-4 h: 6.08 mg L^{-1}), theront (EC_{50}-3 h: 46.80, EC_{50}-6 h: 31.42 and EC_{50}-12 h: 11.83 mg L^{-1}), and *Argulus* sp. adults (EC_{50}-9 h: 61.29, EC_{50}-12 h: 39.04 and EC_{50}-15 h: 27.18 mg L^{-1}) (Figure 1).

For *Argulus* sp. Juveniles, EC_{50} values were 3.65, 1.26, and 7.1 mg L^{-1} lower than those for adults (Table 2S). The concentration of azadirachtin and exposure time as 25 mg L^{-1} for 4 h, 50 mg L^{-1} for 6 h, and 100 mg L^{-1} for 12h against *Dactylogyrus* sp., *I. multifiliis* theront, and *Argulus* sp., respectively, were selected as RAE (Figure 2).

The effect of azardirachtin treatment on morphological features of *Dactylogyrus* sp., *I. multifiliis* theronts, and adult *Argulus* is shown in Figure S5, S6, and S7, respectively.

3.3.2. In Vitro Ovicidal Effect of Azadirachtin on ArgulusEggs. Data on the in vitro ovicidal effect of azadirachtin on *Argulus* eggs, including cumulative percentage hatching rates and egg conditions before and after 14 days post-treatment, are presented in Figure 3 and Figure S8, respectively.

It can be noticed that control eggs showed complete hatching, while treatment group eggs remained unclear with unhatched nauplii. *Argulus* eggs hatching was inhibited at concentrations above 100 mg L^{-1}. In the control and 25 mg L^{-1} groups, nauplius twitching began on day 6, with hatching commencing on day 8 and reaching 100% by day 14. Hatching ceased across all treatments after day 14, with no further hatching observed in the subsequent 7 days. Less than 50% of eggs hatched at 150 mg L^{-1} and 175 mg L^{-1} within 14 days. Eggs in the 125−175 mg L^{-1} treatments became unclear by day 14, with developed nauplii unable to hatch. Hatched nauplii survived 36−48 h in the control group, 12−24 h in the 25 and 50 mg L^{-1} groups, and less than 6 h in higher concentrations.

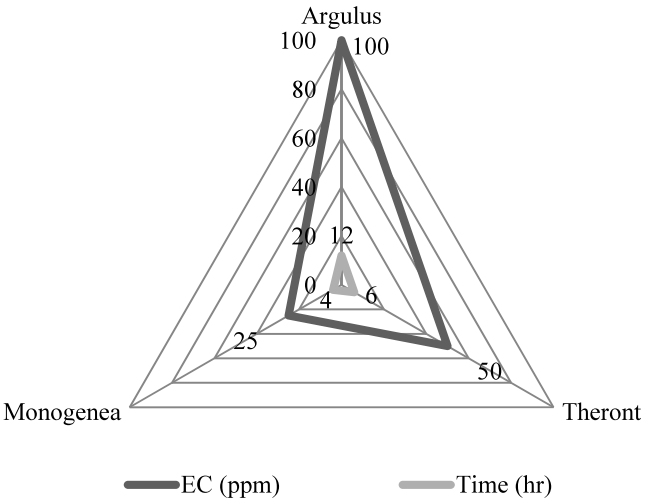

Figure 2. In vitro RAE (100% AE) of azadirachtin with reference to concentration and time for different ectoparasites.

3.4. Acute Toxicity Test of Azadirachtin for Goldfish and TI of Azadirachtin against Each Ectoparasite. The results of acute toxicity tests of azadirachtin (EC 21.5%) expressed in terms of LC_{50} values at different time periods for *C. auratus* were found to be 78.36, 59.47, 25.07, and 20.48 mg L^{-1} for 3, 6, 12, and 15 h, respectively (Figure 1), and the TI was estimated 11.22 at 4 h for *Dactylogyrus* sp., 2.11 at 12 h for *I. multifiliis* theronts, and 0.65 at 15 h for *Argulus* sp. (Figure 4).

4. DISCUSSION

The menace of ectoparasites like *Argulus* sp., *Ichthyophthirius* sp., and both monogenean viz *Dactylogyrus* and *Gyrodactylus* sp. on *C. auratus* are reported from various parts of the globe which cause great damage to the host and hamper the ornamental fish trade.[7,11,39−42] Ectoparasites are mostly attached to the host body surface, affect their free movement, and cause excessive secretion of mucus. The present study also showed that the presence of these parasites on the fish body obstructs their

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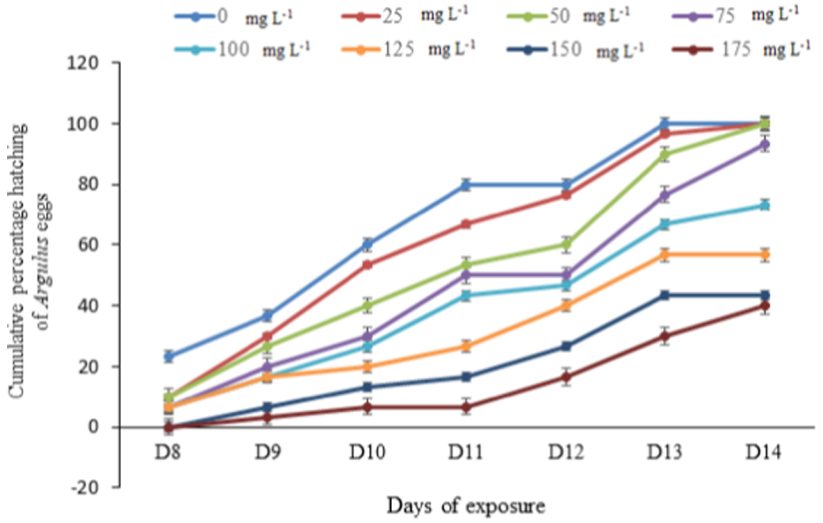

Figure 3. Cumulative percentage hatching of *Argulus* eggs at different time intervals exposed to different concentrations of azadirachtin (EC 21.5%) solution.

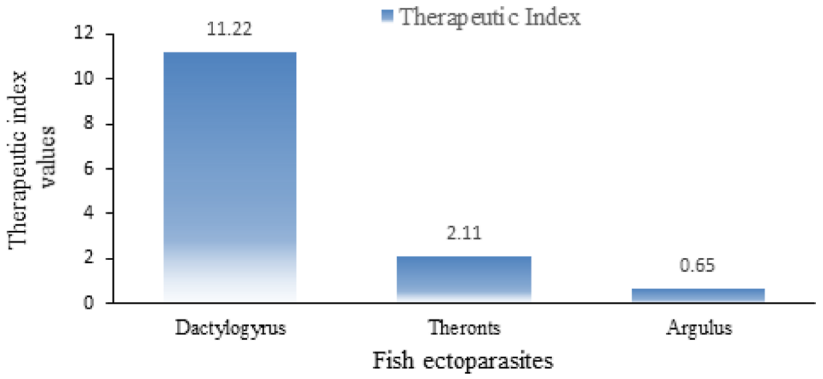

Figure 4. TI value for safe application of azadirachtin against different ectoparasites (*Dactylogyrus*: 4 h, *I. multifiliis* theront: 12 h, and *Argulus*: 15 h) on *C. auratus*.

normal movement and activities. The recorded prevalence of 41.33% for *Dactylogyrus* sp., 35.33% for *Argulus* and 28.67% for *I. multifiliis* in goldfish in the present study is in accordance with several studies. In two similar and interesting studies on *C. auratus* in Iran, one study reported a prevalence level of 82, 49, and 10% for*Dactylogyrus vastator*, *Dactylogyrus* sp., and *I. multifiliis*, respectively,[43] while another study found prevalences of 72 and 6.67% for *Argulus foliaceus* and *Argulus siamensis*, respectively.[44] Similarly, a high prevalence of *Dactylogyrus formosus* in China,[45] and *I. multifiliis*, followed by *Dactylogyrus* sp. in Iran has been reported.[40] Collectively, these reports emphasize that ectoparasites are a significant issue in *C. auratus*.

The concentration of azadirachtin used in treatment of ectoparasites was positively correlated to mortality of parasites, showing a dose-dependent mortality pattern. In *in vitro* test, azadirachtin (EC 21.5%) solution showed differential degree of antiparasitic effects; moreover, the application of azadirachtin ranged from 1 to 125 mg L^{-1} against examined ectoparasites. A similar finding was reported by Ekanem et al., where *in vitro* application of crude and petroleum extracts of*Mucuna pruriens* and *Carica papaya* against *I. multifiliis* showed 35% and 60% effectiveness in 3 h, and 100% mortality of the parasite in 6 h at 100 and 150 mg L^{-1}, respectively.[30] An investigation on *in vitro* AE of neem leaf extracts against *Argulus* sp. revealed that aqueous extracts at a concentration 3.5 g L^{-1} had 100% AE at 6 h, while extracts prepared in different solvents, such as ethanol, methanol, chloroform, and acetone at 2.0 g L^{-1} concentration, had 100, 75, 66.66, and 50% AE, respectively.[46] Further, investigations demonstrated 100% and 91.66% AE of ethanolic and methanolic extracts of neem leaf extracts against copepodid stage of *Argulus* sp. at 1.25 and 1.5 g L^{-1} in 6 h, respectively.[47] A recent study also demonstrated that an aqueous extract of neem leaves at a concentration of 3.0 g L^{-1} significantly decreased the average intensity of parasitic infestation (*Dactylogyrus* sp. and *Centrocestus formosanus*) in *Oreochromis niloticus*.[48] Eguale et al. found *in vitro* anthelmintic activity of Henna, Ayderke, and Amedmado herbal extracts against *Haemonchus contortus*.[49] Mehlhorn et al. evaluated ovicidal effects of a product (Wash Away Louse) composed of neem seed extracts and reported that an incubation time of only 5 min effectively prevented the hatching of larvae, while 93 ± 4% and 76% of the larvae in the untreated controls of body and head lice, respectively, hatched.[50] Similarly, Kumar et al. reported effective dose of azadirachtin and piperine under *in vitro* condition at concentration 15 and 9 ppm, respectively, against adult *Argulus* sp. and estimated 100% AE during exposure for 3 h.[28,29] The antiparasitic effect of ethanolic and aqueous extracts of ginger at concentrations 75 and 200 g L^{-1} was observed, with parasites dying at 65.6 ± 2.8 and 1.8 ± 0.2 min, respectively.[51] This signifies a dose-dependent response, where an increase in the

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extract concentration was associated with reduction in time to parasite immobilization. Further, testing of two flavonoids from root bark of *Morus alba* caused 100% mortality of *I. multifiliis* theronts at a concentration of 2 mg L^{-1} and had a median effective concentration (EC_{50}) of 0.8 ± 0.04 mg L^{-1} against the theronts.[52] Malheiros et al. also reported the dose-dependent antiparasitic effect of *Mentha piperita* essential oil against *Dactylogyrus cycloancistrium* and *Dactylogyrus cycloancistrioides*, wherein parasites exposed to 160 and 320 mg L^{-1} died within 30 min, while those exposed to 80 mg L^{-1} died after 5 h of exposure.[53]

The result of median lethal concentration (LC_{50}) for *C. auratus* reveals a gradual decrease in dose of azadirachtin with increase in time. Earlier reports indicated similar results, with LC_{50} values of azadirachtin (EC 25%) for *C. auratus* recorded as 98.645, 88.793, and 82.115 mg L^{-1} for 48, 72, and 96 h, respectively.[28] Previously, the 96 h LC_{50} of azadirachtin analogues or similar compounds, such as azadirachtin-enriched extract AZT-VR-K and Margosan-O was reported as 100 mg L^{-1} and 8.8 mL L^{-1} of water for*Lebistes reticulates* and rainbow trout, respectively.[54,55] Winkaler et al. demonstrated that the 24 h LC_{50} of neem leaf extracts for *Prochilodus lineatus* was 4.8 g L^{-1}.[56]

A drug is considered safe and effective for therapeutic purposes when the TI is equal to or higher than 2.[57] The findings of this study ensure the safety of the host organism at lower concentrations. In the case of *Dactylogyrus* sp., since the median EC_{50} was nearly five times less than the toxic dose and the TI was over five, this indicates that it has no risk for the host. Similarly, the TI for theronts at different time points was around or over 2 at 12 h, indicating that it was considered safe for treatment. Though the results of the *in vitro* experiments leading to the mortality of *Argulus* when exposed to azadirachtin are endorsed by a previous report,[28] its application under the in vivo condition is not recommended due to a low TI value (<1.0). It is attributed to the variation in result, which may include factors such as the source of azadirachtin, degree of purification, different grade of product, intrinsic factors like inherent resistance/tolerant potential of host, and extrinsic factors like exposure conditions. The incongruity in EC_{50} suggests a disproportionate response of azadirachtin to parasites as the examined ectoparasites belonged to different groups of phyla and differ in their anatomy and physiology. Interestingly, *Dactylogyrus* was more sensitive and was effectively killed at low concentration of azadirachtin when compared to theront and *Argulus*. The most probable reason for this response owes to the bearing of anatomically protective chitinous coat (carapace) of *Argulus* sp.,[58] the complex cellular organization of theronts with multiple nuclei (2−4 micronucleus and one macronucleus) and a proteinaceous ciliated coat around the cell,[59,60] and the absence of such protective structures in *Dactylogyrus* , which allows greater absorption of chemical molecules.[61] The RAE in the present study suggests that in case of mixed parasitic infestation, the treatment with the dose of 100 mg L^{-1} for 12 h could be able to eliminate the most of ectoparasites including protozoans and metazoans; however, the AE of azadirachtin against these ectoparasites under the *in vivo* condition remains to be evaluated.

Azadirachtin is a potent herbal bioactive molecule obtained from neem (*A. indica*), a triterpenoid belonging to class limonoids and family Meliaceae.[62] Its mode of action in insects might be by its interference with ecdysteroid function because of similarity of structure, hindering moulting and juvenile hormones.[63] The observed effects are crucial for understanding the overt impacts on the whole organism, such as growth inhibition, moulting defects, and sterility;[64] however, these are likely secondary effects resulting from the primary mode(s) of action on dividing cells and microtubule formation.[65] Blocking of cell proliferation and RNA synthesis was also noted after azadirachtin treatment in a protozoan, *Tetrahymena thermophila*.[66] The present *in vitro* test reveals the antiparasitic activity of azadirachtin against both protozoan (ciliate) and metazoan (monogenean and crustacean) parasites, which makes it broad-spectrum in action. Azadirachtin acts as an antifeedant, repellent, and sterility inducer, inhibiting oviposition and sperm production in insects.[67] However, the detailed mechanism of its antiparasitic action in aquatic systems should be further addressed.

The present *in vitro* study clearly demonstrated the broad-spectrum antiparasitic activity of azadirachtin solution in treating common adult protozoan (*I. multifiliis*) and metazoan ectoparasites, such as *Dactylogyrus* sp. and *Argulus* sp., including the juvenile and egg stages of the latter in goldfish. While the TI of azadirachtin (EC 21.5%) indicates its overall safety for antiparasitic use against *Dactylogyrus* sp. and *I. multifiliis* theronts, the development of new delivery systems and dosage forms, along with an *in vivo* and field evaluations, is necessary for its effective practical use against *Argulus* sp., which possesses a unique chitinous carapace.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c10920.

Pilot range-finding dose study for final EC_{50} and LC_{50} test as described in the paper; intensity of *Argulus* on goldfish during artificial infestation challenge study; and AE % of various concentrations of azadirachtin (EC 21.5%) against juveniles of *Argulus* sp. (PDF)

Argulus-infested fish; goldfish infested with *I. multifiliis,* its horseshoe-shaped macro nucleus (400X) and fish mortality; *Dactylogyrus* sp.; *Argulus* egg clutch on different substrates; *Dactylogyrus* sp. before and after treatment with azadirachtin; *I. multifiliis* theronts before and after azadirachtin treatment; adult *Argulus* before and after azadirachtin treatment; and *Argulus* eggs in control and azadirachtin solution on day 1 and day 14 (PDF)

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Notes

The authors declare no competing financial interest.

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