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ORIGINAL PAPER

In vitro and in vivo antiparasitic activity of Azadirachtin against *Argulus* spp. in *Carassius auratus* (Linn. 1758)

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Abstract *Argulus* is one of the most common and predominant ectoparasites which cause serious parasitic disease and is a potent carrier of viruses and bacteria in the ornamental fish industry. In recent years, organic (herbs)-based medicines are widely used to cure the disease, and neem (*Sarbaroganibarini*) medicine is very popular and effective throughout the world. The present study was conducted to find the effects of Azadirachtin against *Argulus* spp. on *Carassius auratus* under in vitro and in vivo conditions. The 96-h median lethal concentration (LC_{50}) for Azadirachtin EC 25% against *Carassius auratus* was found to be 82.115 mg L^{-1}. The antiparasitic activity test under in vitro and in vivo was evaluated at 1 (T1), 5 (T2), 10 (T3), 15 (T4) and 20 mg L^{-1} (T5) to treat *Argulus* for 3 h and 72 h, respectively. In vitro effect of Azadirachtin solution led to 100% mortality of *Argulus* at 20 and 15 mg L^{-1} for 2.5 and 3 h, respectively. Whereas, under in vivo test, the 100% antiparasitic efficacy of Azadirachtin solution was found at 15 and 20 mg L^{-1} for 72 and 48 h, respectively. The EC_{50} for 48 h was 20 mg L^{-1}, and thus, therapeutic index is 4.10. The results provided evidence that Azadirachtin can be used as a potential agent for controlling *Argulus*.

Introduction

Among the bewildering array of the fish parasites, ectoparasites are probably the most significant, infesting almost all the culture system. Several instances of argulosis in different culture systems and fish mortality due to severe infestation of *Argulus* have been reported from different parts of India (Gopalakrishnan 1964; Singhal et al. 1990; Sheila et al. 2002). Crustacean ectoparasites of sub-class brachyuran such as *Argulus* spp. belong to the order Arguloida of the phylum Arthropoda and have been described as economically important pathogens of finfish in temperate and tropical regions (Walker et al. 2004).

Argulus-infested fish show behavioural abnormality, which includes irritation, discoloration, lethargy and anorexia. Further mode of infestation results in puncturing the host's skin, injecting a cytolytic toxin through pre-oral stylet and feeding on blood, besides mucus and epithelial cells (Lamarre and Cochran 1992). The most common substances used to treat *Argulus* parasites on commercial farms are various bath treatments including hydrogen peroxide, dichlorvos and cypermethrin (Pike and Wadsworth 1999; Toovey and Lyndon 2000). However, the threats of bioaccumulation and residual formation in the host caused by the frequent use of these drugs have led to the need of other alterative control methods (Goven et al. 1980; Klinger and Floyd 2002).

In recent years, many traditional plant-based medicines have been used to control bacterial and parasitic infections in humans and animals (Tona et al. 1998; Willcox and Bodeker 2000; Asres et al. 2001; Satrija et al. 2001; Ijah and Oyebanji 2003). However, the use of medicinal plant extracts for the treatment of parasitic diseases in fish has rarely been reported, but the use of medicinal plant extracts as an effective alternative to antibiotics and pesticides is well documented. Phytotherapy has gained importance to combat the disease problem in aqua-farm as well as in ornamental practices due to its efficacy, cost-effectiveness and eco-friendly properties. Recently,

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use of raw extract of garlic (*Allium sativum*) has been reported to kill trichodinids (Madsen et al. 2000) and theronts and tomonts of *Ichthyophthirius multifiliis* (Buchmann et al. 2003). Ekanem et al. (2004), has assessed petroleum extracts of *Mucuna pruriens* and *Carica papaya* showing some potential for killing the free-swimming stages of *I. multifiliis*.

Azadirachta indica A. Jass. traditionally is known as the "Village pharmacy" or "Village dispensary" in India (Biswas et al. 2002). There are many reports about the antimicrobial, nematocidal, biopesticidal and immunomodulatory activities of the plant neem. Forty active principal ingredients are reported from the extract of the plant neem. Among them, tetranotripenoids or, more specifically, Azadirachtin is an important bioactive compound and highly oxygenated triterpenoid, having antimicrobial and pesticidal properties (Govindachari and Gopalkrishnan 1998). It is more over remarkably non-toxic to vertebrates (Butterworth and Morgan 1968). Thus, there has been much interest in recent years regarding the use of various neem products in ayurvedic and herbal medicine, such as fungicidal, anti-inflammatory, anti-ulcer, spermicidal and dermatological effects. Keeping these in view, the study was carried out with the objective of evaluating the antiparasitic effect of Azadirachtin against *Argulus* spp. in *Carassius auratus*.

Method and materials

Experimental animals

Animals used for experimental purpose were mature goldfish (*C. auratus*) with an average weight of 20.75± 0.25 g. The stock was acclimatized under aerated conditions for a period of 10 days and was fed with commercial pelleted goldfish diet at 2% of body weight.

Experimental design and sampling procedures

Artificial *Argulus* infection was carried by cohabitation method (Saurabh and Sahoo 2010). The fish were periodically observed for development of infestation. After 2 weeks of gestation, fishes were randomly selected and checked for the prevalence and intensity of parasites. Fish were chosen for the in vivo tests at the prevalence of moderately infested 15–20 *Argulus*/fish and randomly distributed in fibre-reinforced plastic tanks of 150-L capacity; in vivo bioassay was performed, and *Argulus* was treated off the host in an in vitro test for evaluation of antiparasitic activity of Azadirachtin against *Argulus* spp. The experiment was conducted in triplicate with complete randomized design.

Acute toxicity assay

The acute toxicity of Azadirachtin solution was assayed for the evaluation of their safety to the host. For median lethal concentration (LC_{50}), a static short-term toxicity test was conducted according to the standard method (APHA 2005). To determine the LC_{50} of Azadirachtin for *C. auratus* exposure for first range finding with 0, 1, 10, 50, 100, 150 and 200 mg L^{-1}; second range finding with 20, 40, 60, 80 and 100 mg L^{-1}; and definitive test with 75, 80, 85, 90, 95 and 100 mg L^{-1} of Azadirachtin solution for 96 h along with the control in bore well water was carried out. All three tests were performed as static bioassay test in triplicate keeping 10 fishes in each tank of mean weight (5±0.50 g). No feeding was done during the experiment, and percentage mortality was recorded at 24, 48, 72 and 96 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. Data obtained from the experiment were processed by Probit analysis, and the graphs were obtained using SPSS graph sheet.

Preparation of stock solution and working test solution

Azadirachtin is soluble in polar organic solvent and slightly soluble in water (Morgan 1968). The preparation of stock solution was carried out in organic solvent dimethyl sulfoxide (DMSO) following Ekanem et al. (2004). The commercially available Azadirachtin EC 25% (SOM Phytopharma, Hyderabad, India) was used for preparation of stock solution. The concentration of stock solution having 10,000 mg L^{-1} was prepared by dissolving 4.0 g of Azadirachtin (EC 25%) in 2 ml of DMSO and the volume was made up to 100 ml by adding distilled water. From this stock solution, the different working test solution was prepared by dissolving in the bore well water as 1 (T1), 5 (T2), 10 (T3), 15 (T4) and 20 mg L^{-1} (T5), and the control solution (T0) was made up of water and 2% DMSO solution with no Azadirachtin.

In vitro bioassay

The in vitro test was performed as per (Ekanem et al. 2004) protocol. The lice (*Argulus* spp.) of heavily infested fish was gently picked with the help of plastic forceps and put into a Petri dish and actively moving parasites were selected with the help of a small hairbrush. Ten live active parasites were manually transferred into a Petri dish containing 20 ml of different concentrations of working Azadirachtin test solution. At every 30 min, the numbers of killed parasites were counted. Parasitic death was considered when the organism did not exhibit any movement after

5 min of observation and after a slight touch with a feather forceps. The experiment was conducted with working test solution in triplicate and compared with a control group under the same test conditions with 2% DMSO solution but with no Azadirachtin.

In vivo bioassay

The in vivo test was performed following Wang et al. (2009) with slight modification. Eighteen fibre resin plastic tanks of (80×57×42 cm) dimension, each of 150-l capacities, were arranged with 24 h of aeration facilities in the wet laboratory of Aquatic Animal Health Management Division, CIFE, Mumbai, India. These tanks were filled with 100 l of bore well water. The experimental fishes (*Argulus* infested) were randomly divided into five treatment groups and one control of stocking density of five number per tank. Following the protocol, in vivo evaluation of Azadirachtin was performed by bath treatment using five concentrations, 1 (T1), 5 (T2), 10 (T3), 15 (T4), and 20 mg L^{-1} (T5), respectively, with the control (T0) tank containing 2% DMSO solution without Azadirachtin.

The *Argulus*-infested fishes were subjected to bath treatment with Azadirachtin solution at the above concentrations for three consecutive days. During this period, fish mortality and parasite mortality were recorded. The effectiveness of each treatment was confirmed by the comparison of the average number of surviving parasites in each treatment group with those in the control group after 72 h treatment. Finally, antiparasitic efficacy of each treatment was calculated using the following equation (Wang et al. 2009):

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

Where, AE=antiparasitic efficacy, B is the mean number of surviving *Argulus* in the control and T is the mean number of surviving *Argulus* in treatment.

Statistical analysis

The data were statistically analysed by statistical package SPSS version 16, and acute toxicity test LC_{50} was estimated at the 95% confidence interval with upper confidence limit and lower confidence limit (Finney 1971) by probit analysis; the graph was obtained using SPSS graph sheet.

Results and discussion

Bath treatment with Azadirachtin solution resulted in a significant mortality of *Argulus* spp. in *C. auratus*. Artificial *Argulus* infection was carried out for 2 weeks, and we observed a moderate infection level of 15–20 *Argulus* per fish. Most of the *Argulus* were found to be attached to the caudal and dorsal fin regions of the host, and large-size *Argulus* were prominently seen on the caudal peduncle region. This moderate intensity of parasites caused low level of haemorrhages in some of the goldfish, and no mortality was observed during the experiment. The similar work design was followed by Saurabh and Sahoo (2010), keeping in mind natural field outbreaks where different degrees of infected fish of the same species are available in one environment. Another success was observed by Forlenza et al. (2008) when they collected adult *Argulus japonicus* from stock carp and subsequently after hatching larval lice were held in groups of 150 individuals/beaker under identical conditions, and infestation was carried out to naive healthy fish. *Argulus* attach primarily to the caudal peduncle of carp in culture ponds (Bazal et al. 1969) and found less marked site preference in some argulids. At 28°C, most parasites were found on the flank, caudal fin and pectoral fins, and at lower temperature, many of the large parasites (over 2.8 mm) were found on the surface of the operculum (Schlüter 1987).

The results of acute toxicity tests of Azadirachtin for *C. auratus* expressed in terms of LC_{50} values are 98.645, 88.793 and 82.115 mg L^{-1} for 48, 72 and 96 h, respectively, showing gradual decrease with increase in time as Fig. 1. In laboratory trials conducted by Zebitz (1987), guppies (*Lebistes reticulates*) tolerated up to 100 mg L^{-1} of Azadirachtin-enriched extract AZT-VR-K under static conditions. The 96 h LC_{50} of Margosan-O for rainbow trout was found to be 8.8 ml L^{-1} of water (Larson 1987). In the common carp (*Cyprinus carpio*), the 96 h LC_{50} of Neem Aza-T/S was above 100 mg L^{-1}, i.e. the highest concentration tested (David and Kumar 1996). Winkaler et al. (2007) demonstrated that the 24 h LC_{50} of neem leaf extracts for *Prochilodus lineatus* was 4.8 gL^{-1}, with confidence interval ranging from 3.7 to 6.2 gL^{-1}.

In vitro test of antiparasitic effect of Azadirachtin solution showed that the application of Azadirachtin at 1,

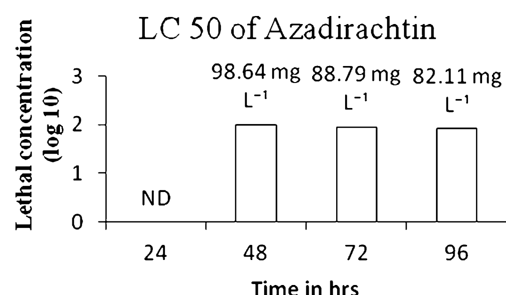

Fig. 1 Acute toxicity of Azadirachtin for *Carassius auratus* for 24, 48, 72 and 96 h

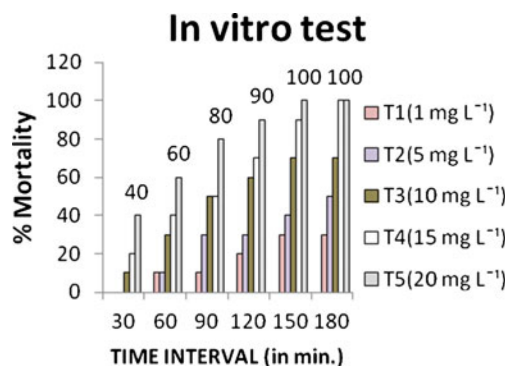

Fig. 2 In vitro mortality of *Argulus* sp. treated with different concentrations of Azadirachtin solution

5, 10, 15 and 20 mg L^{-1} led to mortality of 30%, 45%, 75%, 85% and 100%, respectively, in 3 h (Fig. 2). Azadirachtin concentration was positively correlated to mortality of parasites. A similar finding was reported by Ekanem et al. (2004) where in vitro application of crude extracts of *Mucuna pruriens* and *Carica papaya* against protozoan fish parasite where petroleum extracts of *Mucuna* and *Papaya* showed 35% and 60% effectiveness in 3 h and 100% mortality of parasite was observed in 6 h at 100 and 150 ppm, respectively. Anthelmintic activity of Henna, Ayderke and Amedmado herbal extracts also showed in vitro efficacy against ectoparasites (Eguale et al. 2010). Similarly, Mehlhorn et al. (2011) evaluated ovicidal effects of a product (Wash Away Louse) composed of neem seed extracts against eggs of body and head lice for a different time interval, and he found that an incubation time of only 5 min was effective to prohibit any hatching of larvae, whilst 93±4% and 76% of the larvae in the untreated controls of body and head lice hatched respectively

In vivo study showed that bath treatment with different concentration of Azadirachtin resulted in a significant reduction in the *Argulus* burden of goldfish. The observed reduction of parasites in the test groups could be attributed to the effects of the Azadirachtin because a similar reduction in parasite burden was not observed in the control groups. In vivo test showed that 1, 5, 10, 15 and 20 mg L^{-1} test solutions of Azadirachtin have antiparasitic efficacy of 34%, 56%, 67%, 78% and 100%, respectively, in 48 h, and 46.5%, 71.4%, 78%, 100% and 100% in 72 h, respectively (Table 1). These findings ensure the safety of the host organism, and as the median EC_{50} was nearly four times less than the toxic dose, this indicates that it has no risk for the host. In vivo application of two isolated compounds arctigenin and arctiin against gill parasites had shown to be effective at 0.62 and 3.55 mg L^{-1} after 48 h with 50% and 100% efficacy at 10.0 mg L^{-1} (Wang et al. 2009). Similarly, in vivo evaluation of extracts of *Radix*, *Fructus*, *Caulis*, *Semen aesculi* and *Semen pharbitidis* in different solvents shows 100% efficacy against *Dactylogyrus* (Liu et al. 2010). The results of the in vitro experiments leading to the mortality of *Argulus* have shown a faster effect compare to in vivo. The difference of in vitro and in vivo application is nothing but temporal difference and could be explained by the fact that *Argulus* are vulnerable to treatment in vitro because they are detached from the host, whereas under in vivo conditions, *Argulus* may be protected within the scales and fins of the host. The lack of correlation between in vivo and in vitro tests observed in this study is applicable to all areas of drug research (Kirby 1996). The in vitro assay may be excellent in measuring the intrinsic activity of a substance but cannot possibly emulate complex in vivo situations (Kirby 1996).

Azadirachtin ($C_{35}H_{44}O_{16}$) is a triterpenoid of the class of limonoids, obtained from neem (*A. indica*) belonging to family Meliaceae (Schmutterer 2002). It is chemically interesting because of its complex structure and the challenge its synthesis provided, biologically interesting because it is a feeding deterrent for some insects and a growth disruptant for most insects, and many other arthropods and species in related phyla (Chavan and Nikam 1982). It is, moreover, remarkably non-toxic to vertebrates. Ruscoe (1971) suggested the effect of Azadirachtin on normal development might be an interference with ecdysteroid function because of similarity of structure. Antiparasitic effects of Azadirachtin can be explained as its effect on

Table 1 (In vivo study) estimation of antiparasitic efficacy (in percent) of Azadirachtin solution (bath treatment) against *Argulus*

Treatments	Mean no. of surviving *Argulus* (B)						Antiparasitic efficacy (in %)={B−T}×100}/B					
Time	12 h	24 h	36 h	48 h	60 h	72 h	12 h	24 h	36 h	48 h	60 h	72 h
Control	75	73	73	73	72	71	–	–	–	–	–	–
1 mg L^{-1} AZA	68	61	52	48	40	35	9.33	16.43	28.76	34.24	44.44	46.47
5 mg L^{-1} AZA	64	53	40	32	24	20	14.66	27.39	45.20	56.16	66.66	71.83
10 mg L^{-1} AZA	55	42	35	24	18	15	26.66	42.46	52.05	67.12	75.00	78.87
15 mg L^{-1} AZA	48	32	26	16	10	0	36.00	56.16	64.38	78.08	86.11	100
20 mg L^{-1} AZA	35	28	14	4	0	0	53.33	61.64	80.82	100	100	100

moulting and juvenile hormones. The effects found are very important for defining the overt effects seen in the whole animal on inhibition of growth, moulting defects and sterility (Schlüter 1987); however, their effects are probably secondary ones caused by the main mode or modes of action on dividing cells and microtubule formation in cells (Mitchell et al. 1997). Blocking of cell proliferation and RNA synthesis was also noted after Azadirachtin treatment in a protozoa *Tetrahymena thermophilae* (Mordue and Blackwell 2004). Recent work indicated that the action of Azadirachtin, at the cellular level in actively dividing cells, was to block microtubule formation. However, the detailed mechanism of action regarding the antiparasitic activity in aquatic system of Azadirachtin should be further addressed.

The present study provides a significant basis for use of the Azadirachtin solution at a concentration of 15 ppm for treatment of *Argulus* on *C. auratus*. However, further studies are required for field evaluation in the practical system, and the mechanism of the anti-ectoparasitic (*Argulus* spp.) activity remains to be performed.

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