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Efficacy of neem seed extracts on developmental stages of *Lernaea cyprinacea*

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Abstract

The efficacy of neem (*Azadiracta indica*) against *Lernaea cyprinacea* was studied. Two types of neem seeds powder, *viz*; (1) dried and powdered neem seeds and (2) solvent extracted neem seeds powder prepared by treating dried seeds powder with petroleum ether were used in the present study. These experiments were carried out in the month of August 2014. Different types of extracts were prepared by separately solubilising both types of neem` seeds powder in i) 1% NaCl solution and (ii) water at two temperatures (28 ^{o}C and 90°C). These extracts were tested at varying concentrations (10, 25, 50 and 100 µg mL^{-1}) against *Lernaea*. Results indicated that neem seeds powder treated with solvent and further solubilized with hot water (90 ^{o}C) at a concentration of 100 µg mL^{-1} was effective in preventing the hatching of *L.cyprinacea* eggs and their further development. Nauplii and copepodid-I stages were also exposed to above mentioned neem seed extracts at a concentration of 100µg mL^{-1}. Nauplii became inactive within 2 hrs and copepodids died after 43 minutes exposure to solvent treated neem`s powder extracted with hot water. Fishes (*Labeo fimbriatus)* survived for an average period of 5 and 30 min in all types of neem seeds extracts except the one extracted with water at 28$^{o}$C (both solvent treated and untreated neem`s seeds powder).

Keywords: Nauplii, Copepodid, *Lernaea cyprinacea*, *Azadiracta indica*, *Labeo fimbriatus*.

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Introduction

The ectoparasites of fish constitute one of the most important problems associated with pond fish culture. Lernaeidae is a major family of cyclopoid copepod parasitic groups associated with freshwater fish. The life cycle comprised of three nauplius stages, five copepodid stages and adults (Grabda, 1963). *Lernaea* is often introduced to the pond along with the new fish. This species spread mostly due to lack of adequate sanitary control during fish transport (Boeher and Santos-Neto, 1993). Proper quarantine procedures should help to prevent its introduction into an established pond. Few reports are available on the use ofplant extracts/ herbal products as a parasiticide against *Lernaea* with varying success (Chen, 1933; Anonymous, 1973; Kabata, 1985; Wu yicheng, 1995; Toro *et al*., 2003). Neem (*Azadiracta indica*) has been used successfully in aquaculture systems to control fish predators (Dunkel and Ricilards, 1998) and the aqueous extract of neem`s product is used in fish farms as an alternative for the control of fish parasites (Winkaler *et al*., 2007). However there are no reports on the effect of neem seeds extracts on the developmental stages of the *Lernaea* and its utilization to disrupt the parasite life cycle. The present study explores the possible use of neem seeds extracts as a control measure to disrupt the developmental cycle of the parasite.

Materials and methods

Preparation of neem seeds extracts

Neem (*Azadiracta indica*) seedswere dried and powdered. Two types of neem seeds powder were used for preparing neem seeds extracts. The normal one consisted of dried and powdered neem seeds. The other one, the solvent extracted neem seeds powder was prepared by mixing 10 g of normal seeds powder with five volumes of petroleum ether and the contents was shaken well for 30 minutes. Residues were collected by filtering through Whatman paper no1. The residues were air dried and served as solvent treated neem`s powder for further studies. Different types of extracts were prepared by separately solubilizing 500 mg of both types of neem`s powder in 10 mL of (i) 1% NaCl solution and (ii) water at two temperatures (28°Cand 90°C) and kept overnight with intermittent shaking. Following day, final volume was made up to 50 ml in all the three sets and was individually filtered through filter paper to remove any un-dissolved particles. The final concentration of all the neem seeds extracts prepared as above was 10 mg ml^{-1}.

Efficacy of neem`s seeds extracts on hatching and further development of Lernaea eggs.

The neem seeds extracts prepared as above were added at different concentrations (10, 25, 50 and 100 µg ml^{-1}) to the aqueous culture (70 ml) containing counted number of *Lernaea* eggs obtained from gravid females of *Lernaea*. The eggs counting were done

by examining 0.5 ml of egg suspension under microscope. Counting was repeated for three samples of 0.5 mL and mean value of eggs ml^{-1} were calculated. In the entire set of experiments, on an average of 730 eggs/treatment were used. Same number of *Lernaea* eggs maintained in culture not supplemented with neem seeds extracts served as control. The cultures were daily monitored microscopically for hatching of eggs and death of developmental stages. Counting of nauplii and copepodid-I stages was done by examining 0.5 ml of culture under microscope (10x). Live and actively moving developmental stages, dead and immobile ones are counted separately. Counting was repeated for three samples of 0.5 ml and mean values of nauplii and copepodid-I in total culture (70 ml) was calculated.

Efficacy of neem seeds extracts on developmental stages

In another set of experiment, efficacy of neem seeds extracts on survival of nauplii and copepodid-I stage was studied. Based on the results on the effect of neem seeds extracts on hatching and further development of *Lernaea* eggs, a concentration of 100 µg ml^{-1} was selected. Nauplii and copepodid-I stages were exposed to neem`s seeds extract (both solvent treated, untreated and further solubilized with 1% NaCl, with water at 28°C and 90°C at a concentration of 100 µg ml^{-1}) and were observed under a microscope for immobilization and death of these stages. Death of developmental stages was confirmed by placing them in fresh well water and examining for any sign of revival. The experiment was conducted in triplicate with twenty numbers of developmental stages in each individual treatment. These whole set of experiments were carried out in the month of August 2014. Similarly, tolerance of fingerlings (*Labeo fimbriatus*) (average size 5±1 cm), a peninsular medium carp, to neem seeds extracts was also studied. Fingerlings were exposed to neem seeds extracts at a concentration of 100 µg ml^{-1} (both solvent treated, untreated and then further solubilized with 1% NaCl, with water 28°C and 90°C). For each exposure, 10 fingerlings were used, water quality parameters were maintained as per the requirements for the rearing of fingerlings like pH of 7.5 and temperature of 28°C and etc. The stress/death time of the fish was also recorded.

Statistical analysis

The data was analyzed in completely randomized block design after arcsine transformation (only for Table 1) with analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used to compare means (Gomez and Gomez, 1984). Statistical analyses were performed using AgRes statistical software, version 3.0 for Windows.

Results

Effect of neem seeds extracts on hatching and further development of L. cyprinacea eggs

Results indicated that neem`s seeds powder treated with solvent and further solubilized with hot water (90°C) is

more effective in preventing the hatching of *L. cyprinacea* eggs and their further development when compared to neem`s seeds extracts made from untreated powder. Among all the concentration used in the present study, a concentration of 100 µg mL^{-1} was found to have maximum effect on hatching of *Lernaea* eggs and its further development (Table 1). Hence, a concentration of 100 µg ml^{-1} was selected for further studies.

Table 1: Efficacy of neem seeds extracts on Naupli and Copepod.

Treatment		% Naupli				% Copepod			
		Concentration (µg mL^{-1})				Concentration (µg mL^{-1})			
		10	25	50	100	10	25	50	100
Solvent treated									
T1	1% NaCl	75.33 m	71.00 l	65.67 k	62.33 j	58.67 o	53.67 m	48.33 k	44.33 i
T2	Water 28°C	51.33 h	45.67 g	38.33 e	32.67 c	46.67 j	43.67 i	38.00 g	31.67 d
T3	Water 90°C	35.00 d	31.67 c	27.67 b	23.00 a	33.33 e	28.00 c	24.67 b	20.67 a
Untreated									
T4	1% NaCl	88.00 o	83.67 n	78.67 m	73.00 l	69.67 r	65.67 q	61.33 p	55.67 n
T5	Water 28°C	66.33 k	62.00 j	56.00 i	46.00 g	55.67 n	51.67 l	46.33 j	41.33 h
T6	Water 90°C	43.00 f	37.00 d	32.33 c	27.67 b	40.00 h	35.33 f	30.33 d	25.67 b
	CD (0.05)	Trt (T) 1.26	Conc.(C) 1.02	T X C 2.52		Trt (T) 0.90	Conc.(C) 0.74	T X C 1.80	

* Mean values followed by the same letters are not significantly different according to DMRT (p<0.05). Values were arcsine transformed before analysis

Efficacy of neem seeds extracts on developmental stages

According to results (Table 2), it was found that solvent treated neem seeds powder extracted with hot water has more adverse effect on nauplius and copepodid stages than any other types of extract used in the present study. Nauplii became inactive within 2 hrs and copepodid died after 43 min exposure to solvent treated neem seeds powder extracted with hot water. Nauplii stages showed higher levels of tolerance to exposure to all types of neem`s extracts. The copepodid stages became totally immobilized and were not responding to external stimuli after an average exposure time of 20 min in all treatment groups. However, their internal organ movements were observed after immobilization till their death.

Studies conducted to evaluate the tolerance of fish to neem seeds extracts revealed initial stress reaction in fish exposed to all types of neem seeds extracts, but fishes from all groups recovered quickly. Fish fingerlings have survived an average period of 5 hrs and 30 min in neem seeds extracts which were both solvent treated, untreated and then extracted with 1% NaCl solution and with water (90°C). However, fish survived only for an average of 3 hrs and 50 min when maintained at a concentration of 100 µg ml^{-1} of both types of neem seeds extracted with water at 28°C.

Table 2: Efficacy of neem seeds extract on *Labeo fimbriatus*.

Treatment		Death time		
		Naupli (hr)	Copepod (hr)	Fish (hr)
Solvent treated				
T1	1% NaCl	02:00:00	00:53:33 b	05:47.00 b
T2	Water 28°C	02:00:00	00:35:07 a	03:70.00 a
T3	Water 90 °C	02:00:00	00:43:07 a	05:37:00 b
Untreated				
T4	1% NaCl	02:00:00	01:25:33 d	06:09:00 b
T5	Water 28°C	02:00:00	01:05:33 c	04:07:00 a
T6	Water 90°C	02:00:00	01:29:00 d	05:45:00 b
	CD (0.05)	NS	8.68	0.35

* Mean values followed by the same letters are not significantly different according to DMRT (p<0.05). Values were arcsine transformed before analysis

Discussion

The effectiveness of neem seeds extracts against *Lernaea* probably lies in azadirachtin, which is found in high concentrations in seeds. Zahedi *et al*. (2010) and Rembold (1989) reported that azadirachtin is much concentrated (85%) in the neem seeds. In the present study neem seeds powder treated with solvent and further solubilized with hot water (90°C) was more effective in preventing the hatching of eggs and further development of the parasite than any other types of extracts. This may be because the active components of neem seeds are likely to yield more when treated with solvent. Even though the high temperature could inactivate volatile compounds; it could also increases the release of active compounds and free radicals. Similar observations were made by Majumder *et al*. (1998), El-Mahmood *et al.* (2010) (Cited by Ogbuewu *et al*., 2011), according to whom the active components of neem are slightly hydrophilic, but freely lipophilic and highly soluble in organic solvents. The initial stress shown by the fish upon exposure to all types of neem seeds extracts is in agreement with Winkaler *et al*. (2007) who reported typical stress response of *Prochilodus lineatus* upon exposure to neem leaf extracts. In the present study up on exposure to neem extracted with water at 28°C in both the types (solvent treated and untreated), fish survived up to an average of 3 hrs and 50 minutes. The possible reason for this observation could be that the neem seeds along with major component azadirachtin contain 45% oil (Schmutterer, cited by Zahedi *et al*., 2010) and the presence of volatile compounds could have reduced the survival rate of the fish. However, the concentration used for the present study (*i.e.* 100 µg mL^{-1}) proved to be non-toxic to the fish. This is in agreement with Oyoo-Okoth *et al.* (2011) who reported that extracts of neem are less toxic at low concentrations and concentrations exceeding 3,200 mg L^{-1} influence physiological and biochemical disturbances. This is in further agreement with Mordue and

Blackwell (1993) that neem components are non-mutagenic, biodegradable and non-toxic to mammals.

The present study established the high degree of tolerance shown by nauplii stages of *Lernaea* to neem seeds extracts. The possible reason could be the impermeability of outer membrane or shell of nauplii. However the copepodid stages were susceptible to long term exposure to neem seeds extracts leading to their mortality. The fact that nauplii stages are resistant to the herbal or chemical treatment was further supported by the report of Lahav *et al*. (1964) who found nauplii stages to be resistant to chemical treatments until they moult to the first copepodid stage. The ability of neem seeds extract to kill the copepodid stages of *Lernaea* can be exploited and used as a control measure to disrupt the developmental cycle of the parasite.

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