EVALUATION OF SELECTED HERBAL BIOACTIVE MOLECULES (HBMs) AGAINST COMMON ECTOPARASITE(S) OF FRESHWATER ORNAMENTAL FISH

Dissertation submitted in partial fulfillment
of the requirements
for the degree of

M. F. Sc. (Aquatic Animal Health Management)

by

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JUNE, 2016

Sharma A., 2016. Evaluation of selected Herbal Bioactive Molecules (HBMs) against common ectoparasite(s) of freshwater ornamental fish. M. F. Sc. Dissertation, ICAR-Central Institute of Fisheries Education (University Established Under Section 3 of UGC Act 1956), Versova, Andheri (W), Mumbai-400 061.

Dedicated to,

My Parents, Brother and Sister for their endless love, support and encouragement and Sauravsir for his selfless assistance

केन्द्रीय मात्स्यिकी शिक्षा संस्थान
भारतीय कृषि अनुसंधान परिषद,
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(A university Established Under Sec.3 of UGC Act 1956)
Indian Council of Agricultural Research,
Ministry of Agriculture Govt. of India

Dated : 30th June, 2016

CERTIFICATE

Certified that the dissertation entitled, **"EVALUATION OF SELECTED HERBAL BIOACTIVE MOLECULES (HBMs) AGAINST COMMON ECTOPARASITE(S) OF FRESHWATER ORNAMENTAL FISH,"** is a record of independent bonafide research work carried out by **Mr. ANKIT SHARMA** during the period of study from August 2015 to June 2016 under our supervision and guidance for the degree of **Master of Fisheries Science (Aquatic Animal Health Management)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

I hereby declare that the dissertation entitled **"EVALUATION OF SELECTED HERBAL BIOACTIVE MOLECULES (HBMs) AGAINST COMMON ECTOPARASITE(S) OF FRESHWATER ORNAMENTAL FISH"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

30th June 2016 **(Ankit Sharma)**

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ACKNOWLEDGEMENTS

*First and above all, I praise **God**, the almighty for providing me this opportunity and granting me the capability to proceed successfully. This thesis appears in its current form due to the assistance and guidance of several people. I would therefore like to offer my sincere thanks to all of them.*

*My heart with all its respectful emotions, do take this opportunity to express my deep sense of gratitude and heartfelt thankfulness to my major advisor **Dr. R. P. Raman,** Principal Scientist, Aquatic Environment and Health Management division, ICAR-CIFE, Mumbai, for his valuable guidance, patience, encouragement and support throughout this study and especially for his confidence in me. It has been a privileged pleasure to work under his guidance.*

*I owe my sincere thanks to my advisory committee member, **Dr. Md. Aklakur**, Scientist, Fish Nutrition, Physiology and Biochemistry division, ICAR-CIFE, Mumbai, for his valuable suggestions, motivation, interest and kind support during the course of this study.*

*I gratefully acknowledge the benevolent help of my advisory committee member **Mr. Saurav Kumar**, Scientist, Aquatic Environment and Health Management division, ICAR-CIFE, Mumbai, for his wholehearted help, invaluable support, patience, suggestions, friendliness, care, kind counsel and encouragement for the smooth completion of my work.*

*My sincere thanks to **Dr. Gopal Krishna**, Director, ICAR-Central Institute of Fisheries Education, Mumbai and **Dr. W. S. Lakra**, Former Director, ICAR-Central Institute of Fisheries Education, Mumbai for providing the necessary facilities to carry-out my research work successfully.*

*I wholeheartedly thank to **Dr. K. V. Rajendran**, Head of the Division, Aquatic Environment and Health Management division, ICAR-CIFE, Mumbai and **Dr. P. K. Pandey**, Former Head of the Division, Aquatic Environment and Health Management division, ICAR-CIFE, Mumbai for their support and encouragement.*

*My sincere thanks to faculty of AEHM division, **Dr. K. Paniprasad, Dr. Gaurav Rathore, Dr. S. P. Shukla**, **Dr. Gyatri tripathi**, **Dr. Megha Kadam Bedekar, Mrs. Vidyasree Bharathi, Dr. Kundan Kumar, Mr. Dhamotharan K., Ms. Jeena K., Ms. Rathi Bhuvaneswari, Dr. Sreedharan Krishnan** and **Ms. Husne Banu** for all their help and support.*

*I would like to sincerely thank **Dr. T. S. Nagesh**, Professor, Dept. of Fisheries Resource Management, Faculty of Fishery Sciences, WBUAFS, Kolkata for his constant support, motivation, guidance and showing confidence in me throughout my study period. I would like to thank **Dr. Somen Sahu**, Head of the Division, Dept. of Fisheries Economics, Faculty of Fishery Sciences, WBUAFS, Kolkata for his ideas, assistance and motivation.*

*My sincere thanks are due to the teachers from Faculty of Fishery Sciences, WBUAFS, Kolkata, **Dr. Gadadhar Dash, Dr. T.J. Abraham, Dr. T. K. Ghosh , Dr. S. S. Dana, Dr. S. K. Rout, Dr. R. K. Trivedi, Dr. B. K. Das, Dr. S. Sarkar and Dr. S. Chaudhury** for sharing their knowledge and helping me learn things in a better way.*

I am very much thankful to my seniors ***Stanzin da, Prashant Sir, Abhay Sir, Raju Sir, Sashi Sir, Sanjeev Sir, Shashank Sir, Vimal Sir, Adnan Sir, Aziz da, Renganathan Sir, Biswajit da, Sampa di, Tasok Sir, Mahesh Sir, Raghu Sir, Ambuj da, Baijnath da and Saral da*** *for their help, care, support and encouragement.*

I heartfully thank my bacthmates, ***Mr. Shyam K.U., Mr. Biswajit Das, Ms. Riya P Albin, Ms. Shameena S S, Mr. Lalhmunsanga, Mr. Bharat Kumar V and Ms. Kirti Komal*** *for their wholehearted support, care, inspiration, constant encouragement and suggestions.*

I would like to sincerely thank my friends ***Santhana, Nesnas, Wisdom, G.K., Jaspreet, Raju, Hafeef, Arunava, Suman, Uday, Ratul, Masud, Chandan, Malay, Satyajit, Shiva, Benson and Avinash*** *for their constant support and motivation throughout the study.*

It is with wholehearted love, I express my thankfulness to my dear friends, ***Subhankar, Sunny, Rinchen, Sushant, Passang, Ankit, Tenji, Vishaka, Abhilash, Riyaz and Ravi*** *for their invaluable support, patience and encouragement.*

I am very much thankful to my juniors ***Rahul****,* ***Munish****,* ***Prashanta*** *and* ***Gyandeep*** *for their utmost help, support and motivation throughout my work.*

Words are not sufficient to express my heartfelt love and gratitude to my beloved ***Papa and Mammi*** *for their selfless love, constant support, inspiration, blessings and prayers which kept me in good spirits and paved the way for success in all the endeavors in my life. I thank my dearest brother,* ***Mr. Abhilash Sharma*** *and sister,* ***Ms. Kanchan Sharma*** *for their prayers, support and encouragement that always helped me move in confidence throughout the research especially during my many, many moments of crisis.*

I owe my sincere thanks to ***Mr. A. K. Padmanabhan and Dr. Nalini Poojary*** *for their constant support, help and valuable suggestions during the research period.*

I sincerely acknowledge the ***ICAR*** *for the fellowship during the course of my study.*

My sincere thanks are due to all the staff members of the department and all those who helped me throughout my studies, for their support and help.

30th June 2016 ***(Ankit Sharma)***

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सारांश

जलीय वातावरण में बाह्यपरजीवी सर्वव्यापी हैं जिसकी वजह से ये सजावटी मछलीयो के उद्योग के लिए एक प्रमुख चिंता का कारण है। । अतीत में बाह्यपरजीवी के लिए प्रयोग किए गये रासायन और सिंथेटिक बाह्यपरजीवी नाशक दवावों के कम बुरे प्रभावों के कारण इसे पर्यावरण के अनुकूल और लागत प्रभावी उपचार माना गया है। इस चिंता के निवारण के लिए Phytotherapy (पौधों के अर्क या उसके बायोएक्टिव अणुओं का उपयोग) का उपयोग महत्व प्राप्त कर रहा है। इसलिए, वर्तमान अध्ययन हर्बल बायोएक्टिव अणुओं (Azadirachtin और Allicin) का उपयोग आम बाह्यपरजीवीयो (*Argulus* spp., *Dactylogyrus* spp, *Ichthyophthirius multifiliis*) के खिलाफ मीठे पानी कि सजावटी मछली के कष्ट के ऊपर प्रभावकारिता का मूल्यांकन करने के लिए किया गया था। इन विट्रो वातावरण मे Azadirachtin कि बाह्यपरजीवीयो के प्रतिरोधक प्रभाव का प्रदर्शन 1 -175 मिलीग्राम प्रति लीटर के रेंज में *I. multifiliis* theront, *Dactylogyrus* sp, *Argulus* spp. के वयस्क और किशोर तथा *Argulus* के अंडे के खिलाफ किया गया। इसी तरह, इन विट्रो के शर्त तहत Allicin कि बाह्यपरजीवीयो के प्रतिरोधक प्रभाव का प्रदर्शन 1 से 4 मिलीग्राम प्रति मिलीलीटर के रेंज में *Argulus* spp. और *Dactylogyrus* sp. के खिलाफ किया गया। इन विट्रो Azadirachtin की प्रभावकारिता 12 घंटे मे T_4 (100 मिलीग्राम प्रति लीटर) और T_5 (125 मिलीग्राम प्रति लीटर) में वयस्क और किशोर *Argulus* के लिए, 6 घंटे मे T_5 (47.61 मिलीग्राम प्रति लीटर) मे theronts के लिए और 3 घंटे मे T_7 (30 मिलीग्राम प्रति लीटर) मे *Dactylogyrus* spp. के लिए शत प्रतिशत उपचार पाया गया। सुनहरी मछलियों के लिए Azadirachtin का साधारण घातक सान्द्रता (LC_{50}) 20.48 मिलीग्राम प्रति लीटर 15 घंटे लिए पाया गया और इसकी अनुमानित therapeautic सूचकांक *Dactylogyrus* के लिए 5.33, 1.69 theronts के लिए और 0.65 और 0.69 क्रमशः वयस्क और किशोर *Argulus* लिए किया गया था। Azadirachtin, *Argulus* के T_6 (150 मिलीग्राम प्रति लीटर) और T_7 (175 मिलीग्राम प्रति लीटर) समूह मे के अंडे सेने मे 50% की कमी का कारण बना। इसी तरह, इन विट्रो allicin की प्रभावकारिता T_5 (4 मिलीग्राम प्रति मिलीलीटर) में *Argulus* के लिए 18 घंटे में और T_7 (4 मिलीग्राम प्रति मिलीलीटर) में *Dactylogyrus* के लिए 2 घंटे में था। सुनहरी मछलियों के लिए allicin की LC_{50} 1.63 मिलीग्राम प्रति मिलीलीटर 15 घंटे पाया गया और इसकी अनुमानित therapeautic सूचकांक *Argulus* के लिए 1.36 और *Dactylogyrus* के लिए 5.57 था।Azadirachtin इन विट्रो की शर्तों के तहत Allicin से अधिक शक्तिशाली पाया गया । Therapeautic सूचकांक से पता चलता है कि Azadirachtin *Dactylogyrus* spp. और theronts के इलाज के लिए *in vivo* वातावरण मे इस्तेमाल किया जा सकता है लेकिन *Argulus* संक्रमण के लिए नही। इसी तरह allicin, *Dactylogyrus* spp के खिलाफ सुनहरी मछलियों मे किया जा सकता है लेकिन *Argulus* संक्रमण के लिए नहीं इस्तेमाल किया जा सकता है। Azadirachtin 150 और 175 मिलीग्राम प्रति लीटर सान्द्रण में *Argulus* के अंडे सेने की गति को धीमा करने के लिए भी इस्तेमाल किया जा सकता है। हालांकि, दोनों Allicin और Azadirachtin सजावटी मछलीयो उद्योग में, सुनहरी मछलियों मे बाह्यपरजीवी संक्रामण को नियंत्रित करने में उपयोगी हो सकता है, लेकिन इन बायोएक्टिव अणुओं की कार्रवाई की विधा तथा इन विवो वातावरण मे प्रभावकारिता का अध्ययन प्रतीक्षा में है ।

ABSTRACT

Ectoparasites are ubiquitous in the aquatic environment and is a major concern for ornamental fish industry. Due to the ill effects of chemical and synthetic parasiticides in the past on the host and environment, now it is a call for eco-friendly and cost effective treatments against ectoparasites. Phytotherapy is gaining importance in this concern either through the use of plant extracts or its bioactive molecules. Hence, the present study was conducted to evaluate the antiparasitic efficacy of herbal bioactive molecules (Azadirachtin and Allicin) against common ectoparasites (*Argulus* spp, *Dactylogyrus* sp and *Ichthyophthirius multifiliis*) infesting freshwater ornamental fish. The antiparasitic activity of azadirachtin under *in vitro* condition was performed in the range from 1 to 175 mg L^{-1} against *Argulus, Dactylogyrus, Ichthyophthirius multifiliis* theronts and *Argulus* eggs. Similarly, antiparasitic activity of allicin under *in vitro* condition was performed in the range from 1 to 4 mg ml^{-1} against *Argulus* spp. and *Dactylogyrus* sp. The *in vitro* antiparasitic efficacy of azadirachtin was 100% in 12 h for adult and juvenile *Argulus* in T_4 (100 mg L^{-1}) and T_5 (125 mg L^{-1}) treatment groups, 6 h for theronts in T_5 (47.61 mg L^{-1}) treatment group and 3 h for *Dactylogyrus* in T_7 (30 mg L^{-1}) treatment group respectively. The median lethal concentration (LC_{50}) of azadirachtin for goldfish was 20.48 mg L^{-1} for 15 h and its estimated therapeautic index was 5.33 for *Dactylogyrus*, 1.69 for theronts and 0.65 and 0.69 for adult and juvenile *Argulus* respectively. Azadirachtin caused 50 % reduction in hatching of *Argulus* eggs in T_6 (150 mg L^{-1}) and T_7 (175 mg L^{-1}) treatment groups. Similarly, *in vitro* antiparasitic efficacy of allicin was 100% in 18 h for *Argulus* in T_5 (4 mg ml^{-1}) treatment group and 2 h for *Dactylogyrus* in T_7 (4 mg ml^{-1}) treatment group respectively. The LC_{50} of allicin for goldfish was 1.63 mg ml^{-1} for 15 h and its estimated therapeautic index was 5.57 for *Dactylogyrus* and 1.36 for *Argulus* respectively. Azadirachtin was found to be more potent against ectoparasites than allicin under *in vitro* conditions. The therapeautic index shows that azadirachtin can be used for *in vivo* treatment of *Dactylogyrus* sp. and *I. multifiliis* theronts infesting goldfish but not for *Argulus* infection. Similarly, allicin can be used for *in vivo* treatment of *Dactylogyrus* sp. infesting goldfish but not *Argulus* infection. Azadirachtin can also be used for retarding hatching of *Argulus* eggs at 150 and 175 mg L^{-1} concentration. Though, both allicin and azadirachtin can be useful in controlling ectoparasites infesting goldfish in the ornamental fish industry,but study on the mode of action of these bioactive molecules and *in vivo* antiparasitic efficacy is awaited.

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1. INTRODUCTION

Keeping colourful and fancy fish, popularly known as ornamental fish is one of the oldest and popular hobbies dating back to many centuries. Watching ornamental fish provides aesthetic pleasure while culturing them gives financial openings. Due to the increasing demand of ornamental fish throughout the world, there has been a steady increase in its trade globally. A report from (MPEDA, 2005) says that the annual growth rate of ornamental fish trade is 8% with a turnover of US $ 6 Billion. Developing countries have also become popular in the trade of ornamental fish. India is bestowed with two of the 34 biodiversity „hotspot" areas of the world namely the north eastern regions and the western ghats in the south and is a house to 100 indigenous ornamental fish. The export earnings from ornamental fish trade is US $ 350 million with 60% contribution from the developing countries (FAO, 2007). India contributes a negligible amount of 0.32% ornamental fish in the global trade with 90% of the fish being wild caught and rest 10% cultured. The overall scenario of ornamental fish provides a lot of scope for further development.

Goldfish, *Carassius auratus* is the most popular aquarium fish, which belongs to the cyprinid family and is a native to temperate Europe and Asia and now its trade is throughout the world. To meet the global demands of goldfish, farmers culture them in confined environment under intensive culture system with rudimentary handling. Since ornamental fish are forced to remain under highly crowded and artificial conditions, they are susceptible to a wide range of bacterial, viral and parasitic diseases (Bondad-Reantaso *et al*., 2005; El-Galil and Aboelhadid, 2012) which is a challenge for the industry. Parasites are integral part of the ecosystem and are always associated with wild ornamental fish causing impacting problems even in cultured fish (Scholz, 1999). Among the bewildering array of fish parasites, manifestation of ectoparasites are ubiquitous in the aquatic environment and are calamitous on aquatic animals cultured in ponds and aquaria (Tonguthai, 1997). Ectoparasites of teleost fish range from unicellular protozoan (ciliophores and flagellates) to metazoan platyhelminthes (monogenean), crustacean (copepods and branchiurans) and leeches (hirudenian). The utmost prevalence of ectoparasites and concern for fresh water aquarium fish are

Ichthyophthithrius spp., *Trichodina* spp., *Ichthyobodo* spp., monogenean (*Dactylogyrus* and *Gyrodactylus*), *Lernaea* spp. and *Argulus* spp. (Chanda *et al.,* 2011).

The important issue of parasitism is pathology caused by parasites infesting the host which not only severely restrict the growth and alter the immune-physiology of host but in many a cases produce mortality (Kumar *et al.,* 2012b). Ectoparasites reside on the body surfaces like skin, gills and fins of the host and under moderate and heavy infestations, may cause haemorrhages, anorexia, irritability, ataxia, anaemia, skin necrosis and erosion, dermatitis, detrimental immune reactions (hypersensitivity, anaphylaxis etc.), alteration in condition factor, and many a cases serves as potential source of secondary infections (Hopla, 1982; Nelson *et al.,* 1975; Nelson *et al.,* 1977; Steelman, 1976).

To cope up with the parasitism and their deleterious consequences various chemicals are being used since long for treatment and control of ectoparasites in aquaculture such as salt (sodium chloride), formaldehyde, potassium permanganate, formalin, trichlorfon, emamectin benzoate, powdered quicklime, copper sulphate (Singhal, 1986; Ling *et al.*, 1993; Schlenk *et al.,* 1998; Straus and Griffin, 2002). Moreover traditional parasiticides such as paraziquantel (Schmahl and Melhorn, 1985), mebendazole (Katharios *et al.,* 2006), trichlorfon (Buchmann *et al*., 1987) have been used for controlling monogenean infection. However, the frequent use of these chemical parasiticides has had limited efficacy in reducing ectoparasites infestations and often accompanied by serious drawbacks, including the development of drug-resistant parasites, environmental contamination, and even toxicity to host (Goven *et al*., 1980; Marshall, 1999) which brings in a call for an alternative safe therapy for ectoparasites.

Plants are the miraculous laboratories of nature as they provide various kinds of molecules. In present era, research and development of plant based ectoparasiticidals are given special emphasis. However, the use of medicinal plant extracts for the treatment of parasitic diseases in fish have seldom been reported but the use of medicinal plant extracts as an effective alternative to synthetic chemicals and pesticides is known. Phytotherapy is an excellent alternative multicomponent therapy targeting a

wide variety of pathogens and has gained importance to combat disease problem in aqua-farm as well as in ornamental practices due to its efficacy, cost- effectiveness and ecofriendly properties (Harikrishnan *et al.,* 2009).

Compounds from Garlic have been reported to be effective against *Gyrodactylus turnbulli* (Schelkle *et al*., 2013), Trichodinids (Madsen *et al.,* 2000) and Theronts and Tomont stages of *I. multifiliis* (Buchmann *et al.,* 2003). Azadirachtin has been found to be effective against *Argulus* spp (Kumar *et al.,* 2012b). Piperine from *Piper longam* and methanol extracts of *Piper guineense* have been found to be effective against *Argulus* spp (Kumar *et al.,* 2012a) and monogenea (Ekanem *et al.,* 2004) respectively.

Garlic (*Allium sativum*) a species in the family Alliceace is an edible plant and has been used to combat many disease problem in humans. Garlic consists of bioactive compounds like allicin, alliin, ajoene, diallylsulfide and dithiin which are responsible for its medicinal properties. Allicin (thio-2-propene-1-sulfinic acid *S*-allyl ester) is an organosulphur compound obtained from garlic. It is the main biologically active component of garlic clove extracts. It is an oily, slightly yellow liquid that gives garlic its unique odour. The compound is not present in garlic unless tissue damage occurs, and is formed by the action of the enzyme allinase on alliin. Allicin in its pure form is said to possess antibacterial, antiparasitic, antiviral and antifungal properties (Ankri *et al*., 1999). The main antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes, e.g. alcohol dehydrogenase, thioredoxin reductase, and RNA polymerase, which can affect essential metabolism of cysteine proteinase. Therefore, its biological activity is attributed to either antioxidant activity or thiol disulfide exchange.

The Neem tree (*Azadirachta indica)*, is a member of the Meliaceae family*. A. indica*, *A. Juss*. is traditionally known as the “Village pharmacy” or “Village dispensary” in India (Biswas *et al*., 2002). Neem is chemically rich. Forty active principal ingredients are reported from the extract of the plant neem. Azadirachtin, found only in *Azadirachta* sp. (Capinera, 2008), is a complex tetranortriterpenoid liminoid from the neem seeds. It is an important bioactive compound and highly oxygenated triterpenoid,

having antimicrobial and pesticidal properties (Govindachari and Gopalkrishnan, 1998). Azadirachtin is chemically interesting because of its complex structure; and 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. It is known as the “most potent insect antifeedant discovered till date” (Ishaaya *et al*., 2007). There are many reports about the antiulcer, antifertility, antimalarial, antifungal, antibacterial, antiviral, antioxidant and antiparasitic activities of azadirachtin (Morgan, 2009).

In most of the cases either extract or single bioactive compounds of herbs are being evaluated for their antiparasitic efficacy against parasites or its larval stages. The spectrum of antiparasitism is merely tested and no such literature is available for herbal bioactive molecules against ectoparasites of fish. Keeping these in view, the present research is oriented with following objective.

- To evaluate the Antiparasitic Efficacy (A.E.) of selected Herbal Bioactive Molecules (HBMs) against particular ectoparasites of freshwater ornamental fish

2. REVIEW OF LITERATURE

2.1 Ornamental Fish

Ornamental fish keeping started in China as early as 800 B.C. when goldfish (*Carassius auratus* L.) was kept in aquarium which is one of the most popular and demanded ornamental fish even today (Nasser *et al.,* 2001). The ornamental fish culture is a good economic activity that can provide high profits to the culturists but it has not been properly realized and organized in India and requires scientific study and development (Chanda *et al.,* 2011) as it provides foreign exchange and employment to rural populations (Sharma *et al*., 2011). Ornamental fish culture plays a significant role in the economy of state as well as the country (Ahilan *et al.,* 2010). The world trade of ornamental fish is comprised of around 2500 species and millions of specimens among which 30-35 species are very important and belong to the freshwater habitats (Livengood and Chapman, 2009). 95-96% of the trade is made by freshwater ornamental fishes which are bred in captivity in commercial aquaculture farms (Chapman, 1997). Aquarium fish keeping in India started before independence and is almost about 70 years old (Ayyappan *et al*., 2006). India is bestowed with many natural habitats that are rich in ornamental fishes. The Western Ghats in the southern peninsula and the North-Eastern Region of India are considered to be two of the 34 Biodiversity 'hotspot' areas of the world which along with Lakshadweep Islands, Andaman & Nicobar Islands, Gulf of Mannar, Okha- pin tan, Gulf of Kutch complex, Coast of Kerala, Cape Comorin, Gulf of Mannar and Palk bay is a house to more than 150 species of indigenous ornamental fish and an equal number of exotic ornamental fish (Rani *et al.,* 2014; Madhu *et al.,* 2009). The exotic fish species are most prevalent in the Domestic ornamental fish market of India which is mainly held by home hobbyist (99%) rather than by research institutes or public aquaria. The global trade of ornamental fish is a multibillion dollar industry which is growing at the rate of 8% annually. About 120 countries import ornamental fish among which the largest importers are European Union, U.S.A, Japan, Germany, UK, France and Singapore while majority of supplies comes from the Asian countries with Singapore, Thailand, Indonesia and Czech

republic being the top four exporters of ornamental fish along with China, Philippines, Sri Lanka, Thailand, Taiwan, India and others. Since the inputs required in ornamental fish culture is less and the operating culture system is easy, ornamental fishes are cultured either in ponds or at home in small aquaria (Chapman, 2000). Goldfish, Angelfish, guppies, mollies, barbs, eels, loaches, catfishes, gobies etc. are some of the most important ornamental fish contributing to the international trade (Rani *et al.,* 2014). Out of the many ornamental fish being maintained in captivity, the four major groups of fishes are: (i) Marine tropical species; (ii) Koi and Goldfish; (iii) Freshwater tropical species; and (iv) Freshwater temperate species (Rosenthal *et al*., 2008).

2.2 Goldfish

Goldfish (*Carassius auratus*) belongs to the cyprinids group of fishes and is a native to temperate Europe and Asia (Bnrescu*,* 2004). According to the IUCN 2013 assessment report, Goldfish lies under the category of Least Concern of the Red List and its Taxonomic position is:

Kingdom: Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Cypriniformes

Family : Cyprinidae

Genus : *Carassius*

Taxon name : *Carassius auratus* (Linnaeus, 1758)

Water bodies with slow flowing water or stagnant water forms suitable dwelling place for Goldfish (Man and Hodgkiss, 1981; Etnier and Starnes, 1993). The fish can survive in both eutrophic freshwater and brackishwater bodies including vegetated

ponds and canals (Kottelat and Freyhof, 2007). Goldfish is an omnivore species and feeds on a large variety of food like plants, small crustaceans, insects, detritus etc. (Kottelat and Freyhof 2007). The fish is oviparous and produces sticky eggs that attach to the vegetation and the hatched larvae are pelagic. Spawning usually occurs at 15 – 20 ^{0}C between individual female and few males but juveniles require higher temperature for their growth. The average survival of goldfish in artificial environments is up to 20 years and therefore it is valued as an ornamental fish for ponds and aquaria.

The capability of Goldfish to spawn easily in captivity, take both natural and formulated feeds and survive under poor water quality parameters (Van Der Kraak *et al*., 1992; Kobayashi *et al*., 2002; Stacey, 2003) makes it an important candidate not only for ornamental fish trade but also as a biological model for edible carps (Sokolowska *et al.,* 1984).

2.3 Biodiversity of Common Ectoparasites in Ornamental Fish

Ectoarasites are ubiquitously present in the aquatic environment and are of great concern in aquatic animals raised in ponds and aquaria as they cause severe damage to the host (Tonguthai, 1997; Kumar *et al*., 2012b). They inhabit the skin, fin or gills of fishes for various periods (Hopla *et al*., 1994) with some parasites being host specific while others have the ability to parasitize a large number of host. The vast majority of ectoparasites are invertebrates and under heavy infestations they may cause anaemia, irritability, skin necrosis, dermatitis, detrimental immune reactions (Hypersensitivity, Anaphylaxis etc.), low weight gains, and secondary infections (Hopla, 1982; Nelson *et al,.* 1975; Nelson *et al.,*1977 and Steelman, 1976). Ectoparasites of teleost fish range from unicellular protozoan (ciliates and flagellates) to metazoan platyhelminthes (monogenean), crustacean (copepods and branchiurans) and leeches hirudenian.

Protozoan parasites are unicellular organisms which are found most frequently in the aquatic environment. They have a direct life cycle and have the ability to cause serious damage to the host in case of high proliferation and crowded culture systems (Klinger and Floyd, 1998). Ciliates are the most common of the protozoan ectoparasites that infect ornamental fish and can be identified by the presence of cilia on its body

which is used for locomotion or feeding. They exhibit high degree of specificity and seasonality. *Trichodina, Ichthyophthirius, Epistylis, Apiosoma, Scyphidia, Oodinium and Chilodonella* are the major protozoan ciliated ectoparasites (Tonguthai, 1997).

Protozoan flagellates causes both internal and external infestation in fish. They possess whip like flagella that assist them to move in their typical jerky movement. *Ichthyobodo* is an important flagellate parasite infesting both marine and freshwater fish. They mainly parasitize on the skin and gills and cause steel-gray discolouration of the host body due to copius mucus secretion (Klinger and Floyd, 1998). Other important flagellate parasite include *Hexamita, Piscinoodinium* and *Cryptobia*.

Monogenean ectoparasites are the largest group of metazoan parasites commonly found on the gills, fins and skin of cyprinid fishes causing significant pathology (Galli *et al.*, 2005; Ogawa and Egusa, 1979; Woo 2006). *Dactylogyrus spp* and *Gyrodactylus spp* are the most common fish ectoparasite among this group that infect the gills and skin of the host respectively. They have a direct life cycle and are highly host and site specific (Klinger and Floyd, 1998).

Among the ectoparasites very few information is available about parasitic crustaceans as they are very poorly understood, except by a few specialists (Kabata, 1985). There are a large number of crustacean parasites inhabiting the skin and gills of ornamental fish. The 'fish louse', *Argulus* spp is the most common crustacean ectoparasite of ornamental fish. Ectoparasites not only cause economic losses by killing the fish but also through reducing the market value of the fish.

2.3.1 *Ichthyophthirius multifiliis*

The disease called 'Ich' or 'White Spot Disease' has been a serious problem in aquarium fishes for generations thereby causing economic damage by affecting the freshwater ornamental fish trade (Matthews, 2005). It is caused by a ciliate parasite *Ichthyophthirius multifiliis* which is the largest protozoan parasite of freshwater fish and are among the most common parasites of fish in tropical and temperate regions of the world (Abowei *et al.,* 2011; Buchmann *et al.,* 2001; Klinger and Floyd, 1998) that are

capable of destroying the entire fish population if left untreated (Tucker and Robinson, 1990). They mainly infect the skin, fin and gills of fish causing restlessness, emaciation, body irritation, and respiratory compromise in case of heavy infestation (Schaperclaus, 1991). The parasite is easily recognized by the presence of a horseshoe shaped macronucleus and beating cilia around the body when observed under a microscope but this is not the only character for its identification.

The life cycle of *I. multifiliis* has been well documented (Lom and Dykova, 1992) consisting of three developmental stages: A host associated trophont, a reproductive tomont and infective theronts. The theronts invade the host tissue (fin, skin and gills) and grows into a mature trophont which is visible to the naked eye after some days as white spot. The spreading of the parasite can be prevented by killing the free swimming stages (theronts and tomonts) by the use of various antiprotozoal drugs (Tucker and Robinson, 1990; Schaperclaus, 1991; Straus, 1993).

2.3.2 Monogenea

Monogenean ectoparasites such as *Dactylogyrus* spp*.* and *Gyrodactylus* spp. are the largest group of metazoan parasites commonly found on the gills of cyprinid fishes causing significant pathology (Galli *et al*., 2005; Ogawa and Egusa, 1979; Woo 2006). Heavy infestations of Dactylogyrids are common in both cultured and wild fishes causing morbidity and mortality of the host (Reed *et al.,* 2009). Heavy infestation of monogenean may occur frequently in crowded condition with fishes showing signs of increased respiration, less tolerance to low oxygen level, swollen pale gills and mortality as well as secondary infection by bacteria, fungus or virus may occur (Roberts, 2001).

Transmission of the parasite occurs through physical contact between fishes in some cases and mainly through the parasitic oncomiracidia or larvae. The sudden increase in the population of monogenean is due to their direct life cycle. The adults of both *Dactylogyrus spp* (oviparous) and *Gyrodactylus spp* (viviparaous) are hermaphrodites. The adult *Dactylogyrus* parasites lay eggs directly into the water and then the hatched larvae seeks for a host while the Gyrodactylids directly release larvae

into the water that are capable of attaching either to the same host or a different one (Reed *et al.,*2009).

The anterior end of the monogenean parasites consist of an oral sucker with varied number of marginal hooks and the posterior end consists of an organ which helps the parasite attach to their host. *Dactylogyrus extensus* (Gill flukes) commonly infects the fancy ornamental fish while koi is mainly infected by *Gyrodactylus kathareneri* (skin flukes) and *Gyrodactylus bullatarusdis* and *Gyrodactylus turnbulil* are guppy fish flukes (Woo, 2006; Roberts, 2010).

2.3.3 Crustacea

Argulus spp an ectoparasie of fish, commonly known as the fish lice are members of the branchiuran group of crustacea. They belong to the Arguilidae family of the order Arguiloida and subclass branchiura and is an economically important crustacean ectoparasite of fish (Walker *et al.,* 2004). There are more than 100 species of *Argulus* distributed worldwide (Steckler *et al.,* 2012) but the three species viz. *Argulus foliaceus, A. japonicus*, and *A. coregoni* are prominent in the freshwater fish culture systems and are the most studied. Several instances of *Argulus* infestation in fish culture system and associated mortality has been reported from different parts of India (Gopalakrishnan, 1964; Singhal *et al*., 1990; Sheila *et al*., 2002). *Argulus spp* may be seen with naked eyes attached to the host body since they are 5-10mm in size and the body is divided into head, thorax and abdomen. The body of the parasite is dorsoventrally flattened consisting of a hard exoskeleton, compound eyes, a suctorial proboscis, 2 anteroventral prominent sucking discs that serve as attachment organs and 4 pairs of thoracic swimming legs (Baker, 2008). *Argulus spp* causes direct damage to the host through their attachment and feeding behaviour and indirectly as vectors of other pathogens like nematodes (Sahoo *et al.,* 2013; Moravec, 1994). *Argulus* also acts as vectors for *Rhabdovirus* (Avenant-Oldewage, 2001). Fishes infested with *Argulus* shows signs of irritation, body discolouration, behavioural abnormality, lethargy and reduced feeding. Further damage is caused to the host as the parasite punctures the

skin and injects a cytolytic toxin, thereby feeds on the blood, mucus and epithelial cells of the fish (Lamarre and Cochran, 1992).

Male and female parasites can be identified by the presence of testes and spermathecae respectively due to the transparency of the body in case of live specimens (Flick and Weigertjes, 2005). Mating usually occurs on the host and then the female lays strings of eggs on a hard substratum. Each egg string consists of 1-6 number of rows and 5 -226 eggs which are covered by a gelatinous material. Hatching of the eggs is temperature dependent viz. 10 days at 35^0 C and 61 days at 15^0 C (Steckler and Yanong, 2012). The hatched nauplius survives for 2 days on its yolk reserve but after that it requires a host for completing its life cycle (Avenant-Oldewage, 2001).

2.4 Control and Treatment Measures for Ectoparasites of Fish and their Limitations

Among the bulk of chemotherapeautic agents available for ectoparasitic control and treatment, very few are licensed and safe. Very few research have been done in finding antiparasitic agents for fish ectoparasites among which most of the research have been conducted in the coldwater fish species (Salmonids) and hence the information is extrapolated for use in the treatment of warmwater fishes. Since the treatment conditions such as environmental conditions (temperature, pH, stability, toxicity to other aquatic animals) and individual host factors (safety, metabolism, stress, residues, etc) are entirely different among the coldwater and warmwater, the exchange of treatment information can cause serious problems. These are the reasons that make it difficult to control and treat ectoparasites in commercial situations (Athanassopoulou *et al*., 2009).

However, the frequent use of these chemical parasiticides has had limited efficacy in reducing ectoparasites infestations and often accompanied by serious drawbacks, including the development of drug-resistant parasites, environmental contamination, and even toxicity to host (Goven *et al*., 1980; Marshall, 1999).

A promising drug or therapy is not available for completely eliminating any of the mentioned ectoparasitic infections in fishes. It is better to prevent or reduce the entry of the parasites into the culture systems by following quarantine. In case of freshwater ornamental fish culture system, it is better to give a salt dip to the fish to be stocked followed by three weeks of quarantine, prior to releasing the fish in the culture system. This would assure the reduction in the risk of getting parasitic infestations. Regular observation should be done while the fishes are in quarantine including checking of gill and skin biopsies and in case of any parasitic proliferation specific treatments can be followed at this stage (Reed *et al.,* 2009).

Chemotherapeautic agents are most widely used against ectoparasitic infestations but there is a lack of a broad spectrum, safe and effective agent that can be approved for use in fish culture. Some of the common agents used for controlling and treating ectoparasites in fishes are listed below (Table 1):

Table 1: Commonly used therapeautic agents against ectoparasites of fish.

Sl No.	Ectoparasites	Common therapeautic agents	References
1	**Protozoan fish ectoparasites**	Chloramines-T Formaldehyde, Potassium permanganate, Acetic acid, Copper sulfate, Malachite green, Iodophores, Hydrogen peroxide and salt	Stoskopf, 1993; Noga 2001; Kayis *et al.*, 2005; Balta *et al.,* 2008; and Athanassopoulou *et al.,* 2009
2	**Metazoan fish Parasites**	Levamisole, Mebendazole, Trichlorphon Benzimedazole, Niclosamide, Bithionol and formalin	Lasee, 1995; Toksen, 2006 and Athanassopoulou *et al*., 2009
3	**Crustacean ectoparasites**	Quinine hydrochloride, Atebrine, Potassium permanganate, Dimilin, Trichlorphon, Salts, Organophosphates, Pyrethrins, Avermectin and related drugs	Koyuncu *et al*., 2010 and Athanassopoulou *et al.,* 2009

2.5 Phytotherapy

2.5.1 Brief history

Plants and their products are being used to cure and prevent ailments since time immemorial. In India the first known written record of plant as medicine is in 'Rig Veda' which was written between 3000 to 1800 B.C. Another detailed information on collection and use of plant based drugs is given in 'Atharva Veda'. It was in between 1000 to 600 B.C. plants gained importance as a source of medicine because of the publication of 'Charak Samhita' and 'Sushruta samhita' which provides a detailed information on medicinal plants. Plants as medicine possess the advantage of not bearing any side effects or cumulative toxic effects which is the reason they are being utilised since remote past.

Plants possess or produce many secondary metabolites like pyrethrins, alkaloids, flavonoids, glycosides and steroids that have immense potential in the development of drugs. The use of secondary metabolites for treating ailments has resulted in the development of 'Phytotherapy' (Agarwal, 2002).

2.5.2 Herbs in aquaculture

Various plant extracts are being used in Aquaculture as feed, immunostimulants, antibacterial, antiparasitic, antiinflamatory agents, growth stimulator etc. because of its easy availability, lack of side effects, eco-friendliness, cost effectiveness as well as medicinal properties. Garlic, Neem, Ginger, Papaya, Guava, Aloe vera, Tulsi etc. are commonly used herbs for the control and treatment of different Pathogens of fish along with maintaining a sustainable production.

In a study by (Shalaby *et al.,* 2006) it was found that the food intake, specific growth rate and final weight gain of Nile tilapia increased when it was fed with garlic supplemented feed. Several studies show that intraperitoneal injection or oral administration of various herbal extract stimulates the immune system resulting in increased lysozyme activity, complement activity, phagocytic activity, respiratory burst

activity and other immune parameters. Various studies show that plant extracts are effective against fish pathogens (bacteria, virus, fungus, helminthes and other parasite) both *in vitro* and *in vivo* (Reverter et al. 2014).

2.5.3 Herbal extracts and active ingredients

Herbal extracts refers to the preparations made from the parts of plants such as leaves, roots, bark, fruits etc. which consists of a cocktail of ingredients, while active ingredients are single compound present in the plant that have biological activities. There is still confusion whether herbal extracts are better or their active ingredients. Pharmaceutical demands for the use of single compounds because the dosage can be quantified easily and also it is possible to patent a single compound. On the other hand herbalists believe that since extracts consists of multiple compounds, they tend to increase the therapeautic effects and also dilute the toxicity (Elumalai and Eswariah, 2012). (Dasgupta *et al*., 2010) reported some economicaly important herbs with their active ingredients that can be used for treating ectoparasitess in fishes. Some of the herbs are and their active ingredients are given in (Table 2).

Table 2: Commonly used medicinal herbs and their important active ingredients (bioactive molecules)

Herbs	Active ingredient
1. Ginger	Zingerone, Shogaols and gingerols
2. Turmeric	Curcumin
3. Neem	Azadirachtin
4. Garlic	Allicin
5. Tulsi	Bornylacetate and Cadinene

Thus, every herb consists of a bioactive ingredient in it that provides it various antimicrobial properties.

2.5.4 Phytotherapy against fish ectoparasites

Previously, work has been done using plant extracts or their active ingredients against ectoparasites on fish.

It was found that the methanolic extracts of *Piper guineense* showed antihelminthic activity against monogenean parasites with the presence of three active ingredients (Ekanem *et al.,* 2004).

(Wang *et al.,* 2008) reported that sanguinarine from *Macleaya cordata* is effective against *Dactylogyrus intermedius.* Bruceine A and Bruceine D isolated from dried fruits of *Brucea javanica* was found to be effective against *D.intermedius* with EC_{50} concentrations of 0.49 and 0.57mg/L respectively (Wang *et al.,* 2011).

(Wang *et al.*, 2008) reported that Osthol and Isopimpinellin extracted from the fruit of *Fructus cnidii* was100 % effective against *Dactylogyrus intermedius* in goldfish at concentrations 1.6 and 9.5 mg/ respectively thereby showing antihelminthic activities.

Ginger was found to be effective against *Trichodina spp* and *Epistylis spp* in *Clarias gariepinus* at a concentration of 20mg/L (Abo- Esa, 2008).

(Yi *et al.,* 2012) tested 30 medicinal plants against the protozoan parasite *I. multifiliis* and reported that among the tested plants *Magnolia officinalis* and *Sophora alopecuroides* were most effective with their repective LC_{50} for 4 hrs being 2.45 and 3.43 mg/L.

Similarly, (Kumar *et al.,* 2012a) reported that piperine, an active ingredient of *Piper longum* was effective against *Argulus spp* both *in vitro* and *in vivo* with a therapeautic index of 5.8.

(Ling *et al.,* 2013) showed that the methanol extracts of *Psoralea corylifolia* was able to cause 100% mortality of *Ichthyophthirius multifiliis* theronts *in vitro* at a concentration of 1.25 mg L^{-1} or more in 4 hrs. It was also reported that prolonged

immersion at 5 mg L^{-1} resulted in failure of reproduction of tomonts that were exited from fish treated in bath treatment.

2.6 Azadirachtin (A bioactive molecule of Neem; *Azadirachta* sp.)

Azadirachta. indica, A. Juss. 1830 (synonyms *Melia azadirachta* L. 1753, *Melia indica* (*A. Juss*.) Brandis (1874) *Antelaea azadirachta* (L.) Adelb.), commonly known as the neem tree is a native of Indian subcontinent and is widely distributed in different parts of the world (Schmutterer, 2002). Since age long, different parts of the neem tree are being used for different purposes like, twigs are used for cleaning teeth, timber is used as firewood, for making furniture and fences, neem cake i.e. the leftover after extraction of oil from neem seed is used as an organic manure and the resin is used as 'neem glue'. There are more than 300 secondary compounds associated with the neem tree (Koul and Wahab, 2004). Most of the compounds extracted from the fruits, leaves, seed, bark, roots and flowers belong to the triterpenoid group of compounds (Morgan, 2009). The seeds of azadirachtin consists of 1-3 kernels that are rich in azadirachtin and its homologues (Mordue *et al.,* 2000)

Among the triterpenoids extracted from the neem tree, azadirachtin is considered as the most bioactive and commercially popular one because of its interesting structure and properties (Morgan, 2009). Azadirachtin is extracted from the seeds of the neem tree and its spectral, chromatographic and chemical properties makes it a member of the tetranortriterpenoids which is similar to the compounds nimbin and salannin (Johnson *et al.*, 1996; Morgan, 2009). Out of the 25 natural analogues of azadirachtin, the most bioactive and similar in structure is Azadirachtin A (Schmutterer, 2002; Devakumar and Kumar, 2008). Azadirachtin constitutes about 0.1-0.9 percent or 4-6g/kg of the seed of neem depending on the ecotype of the tree and local conditions (Mordue *et al.,* 2000; Koul and Wahab, 2004).

2.6.1 Structure of azadirachtin

It took about 18 years to develop the structure of Azadirachtin (Morgan, 2009).The structure of azadirachtin includes 16 stereocentres, seven of which are quaternary and nine are secondary, 16 oxygen atoms that are arranged in four ester groups, also, two hydroxyl group, a hemiacetal, an epoxide and a dihydrofuran group is present(Butterworth *et al.*, 1972).

Azadirachtin A, C35H44O16

Among the triterpenoids, azadirachtin is considered as the most oxygenated one isolated so far (Morgan, 2009).

2.6.2 Mode of action of azadirachtin

The effect of azadirachtin have been largely studied against insects and a wide array of effects have been observed that can be broadly included under effects on feeding, physiology and reproduction of the insects. According to Mordue and Blackwell (1993) azadirachtin acts on insects by (i) causing antifeedancy by showing effects on deterrents and chemoreceptors, (ii) blocking morphogenetic peptide release (e.g. PTTH; allatotropins) , resulting in abnormal ecdysteroid and juvenile hormone titre and (iii) directly affecting most other tissues involved in maintaining the proper morphological and physiological characteristics of the insect.

2.6.2.1 Effects on feeding habits of insects

Antifeedency *i.e.* inhibition of feeding in insects is caused due to exposure of azadirachtin. It is proposed that azadirachtin blocks the input from chemoreceptors which normally respond to phagostimulants and hence, if we are able to increase the quantity of phagostimulants, the antifeedancy effect of azadirachtin can be overcome (Chapman, 1974; Schoonhoven, 1982). The deterrent cells of chemoreceptors are stimulated by azadirachtin and at the same time firing of sugar receptors cell is blocked which is responsible for stimulating the feeding activity of the insect (Blaney *et al.,* 1990; Simmonds *et al*., 1990; Mordue *et al.,* 2000). Thus, this results in starvation of the insect and causes its death. The antifeedency effect depends on various factors such as concentration of azadirachtin, methods of application and species of test insects (Mulla and Su, 1999). The ED_{50} dose of neem products causing Antifeedency in different insect groups are Lepidoptera (<0.001-50ppm), Coleoptera (100-500ppm), Hemiptera (100-500ppm), Hymenoptera(100-500ppm) and Orthoptera (0.001- >1000ppm) (Mordue and Blackwell, 1993).

2.6.2.2 Effects on Physiology of insects

The insects treated with neem based products show abnormal moulting, reduced growth, delayed moulting and increased mortalities which is due to the failure of endocrine system controlling growth and moulting (Nisbet, 2000). The moulting is affected because of the inability to produce ecdysteroids (Moulting hormone) and other class of hormone by the insect (Nisbet, 2000).

2.6.2.3 Effects on reproduction of insects

An important effect of azadirachtin against insect is the problem associated with its reproduction. This mechanism can be understood from the previous fact i.e. disruption of ecdysteroids which play an important role in reproduction as it regulates vitellogenesis in insects. Azadirachtin disrupts the ecdysteroids thereby affecting the fecundity, ovarian development and fertility of the insects (Su, 1999). Azadirachtin has

potential effects against insect such as antifeedency, growth regulation, fecundity suppression and sterilization, oviposition repellency and attractancy and biological fitness which makes it suitable for treatment against pest and insects (Su, 1999).

2.6.3 Commercial neem formulations (CNFs) in market

Azadirachtin in markets are available with different names since long for farmers but the concentration varies in all cases. Nimbecidine (Aza 0.03%), Econeem plus (Aza 1%), Soluneem (Aza 6.5%), Limonol (Aza 0.03%), Neemgold (Aza 0.15%), Econeem (Aza 0.3%), Econeem plus (Aza 1%), Fortune Aza (Aza 3 %), Neem Azal-F (Aza 5%) are different neem based products commercially available in India (Kumar *et al.,* 2003). Neemgard, RD-9 Repelin and Neemark are other CNFs available in Indian markets. The Eenvironmental Protection Agency (EPA) has granted registration to Margosan- O, a neem based product in USA for use in crop and ornamental. Azatin EC4.5, Azad WP10, Azad EC45 and Neemix EC0.25 are neem formulations available commercially in Columbia (Su, 1999).

Neem based products are available in the market for use as an insecticide but recently some interest has been gained towards its use in Aquaculture.

2.6.4 Use of Azadirachtin and neem based products in Aquaculture

Neem has been used successfully in aquaculture system to control fish predators (Dunkel *et al*., 1998) and pathogenic bacteria such as *Aeromonas hydrophila*, *Pseudomonas fluorescens, Escherichia coli* and *Myxobacteria spp* (Das *et al*., 2002). Aqueous extracts of neem leaf and other neem based products are widely used in fish farms for controlling fish parasites and Dragonfly larvae which are Fish fry predators (Martinez, 2002).

Kumar *et al.* (2012b) reported that Azadirachtin EC 25% was 100% effective against *Argulus sp.*infecting *Carassius auratus* at a concentration of 15 and 20mg/L. Mordue *et al.* (2004) reported that treatment with azadirachtin resulted in the blocking of cell proliferation and RNA synthesis in a protozoa *Tetrahymena thermophilae*. (Kumar *et al*. (2012b) reported that treatment of *Argulus* infested goldfish with Azadirachtin

showed improved haemato-serum biochemical profile of the fishes and a significant enhancement of their health condition. Logambal and Michael (2000) observed that there was an inverse relationship between the dose of azadirachtin and the degree of immunostimulation in *Oreochromis mossambicus* (Peters).

In the fish farms neem insecticides have been applied @ 50g/ha and the expected effective concentration was found to be 30µg/L (Kreutzweiser *et al.,* 2004).

Azadirachtin and azadirachtin based products are considered as safe for fish, wildlife and other nontarget organisms because plant based molecules are easily biodegradable and are more targetspecific which makes it suitable for use in aquaculture (Su, 1999).

2.6.5 Acute toxicity of Azadirachtin and neem based products on fishes

Studies for evaluating the toxicity of neem preparations and pure azadirachtin have been done on laboratory animals and nontarget species (Gandhi *et al.,* 1988; Osuala and Okwuosa, 1993., Wan *et al*., 1996;).

The 24 h LC_{50} of *Prochilodus lineatus* exposed to neem leaf extract was found to be 4.8 g/L showing its low toxicity but at the same time it caused functional and morphological changes in the fish (Winkaler *et al.,* 2007). Similarly, Ahmad and Ansari (2011) reported the LC_{50} (72hrs) and LC_{50} (96 hrs) of Azacel (Neem based product) on the embryo and fingerling of *Danio rerio* as 0.006µg/L and 0. 05µg/L respectively, showing embryo are at more risk compared to juveniles. Kumar *et al.* (2011) reported the 96hrs LC_{50} of Azadirachtin on *Heteropneustes fossilis* to be 52.35 mg/L showing that this fish is hardy when compared to other fish species. Kumar *et al.* (2012b) reported that the LC_{50} (96 hrs) of *Carassius auratus* exposed to Azadirachtin was 82.115mg/L. The 96 hr LC_{50} concentration for *D.rerio* exposed to different concentration of a neem based bio- pesticide was found to be 0.22 ml/L indicating that it is toxic to aquatic organisms (Maranho *et al.,* 2014). Davoodi and Abdi (2012) found the 96 h LC_{50} of neemgold for *Cyprinus carpio* to be 75.49 mg L^{-1}.

It is very difficult to compare the toxicity of azadirachtin on different fish species as it depends on factors such as light, water, solvents and emulsifiers used for its preparation (Wan *et al.,* 1996). Thus, a holistic idea regarding the toxicity of azadirachtin on aquatic animals is lacking as huge variations are observed in different fish species.

2.7 Allicin (a bioactive molecule of garlic; *Allium sativum*)

Garlic (*Allium sativum* L. family Liliaceae) is among the best known plant used in cooking as well as in medicine and is originally from Asia. It is also referred to as an 'all healing herb'. The medicinal properties of garlic is well known and has been used by Egyptians, Babylonians, Ancient Greeks, Chinese, Vikings, Indian and Romans since thousands of years. Garlic is a bulb and grows upto 20-70 cm bearing hermaphrodite flowers (Mikaili *et al*., 2013). It is composed of various important compounds that includes seventeen amino acids, at least 33 sulphur compounds, 8 minerals (germanium, calcium, copper, iron, potassium, magnesium, selenium and zinc) and Vitamin A, B and C along with fibre and water (Josling, 2008).

Since, garlic grows beneath the soil, it is always exposed to a wide variety of soil borne pathogens. To protect itself from those pathogen garlic is blessed with allicin which is formed in the bulb rapidly if it gets damaged. In a cross section of a fresh healthy garlic clove, allin and enzyme allinase are present in different compartments but as the clove is damaged by any means, they rapidly react with each other to form 'allicin' which is the main bioactive compound of Garlic. Thus, allicin is not present in an undamaged garlic clove but it is formed to protect the clove if it is damaged (Josling 2008). Cavalitto and Bailey (1944) identified allicin which is the substance providing the peculiar smell to garlic.

Investigators have tried to estimate the amount of allin and production of allicin from garlic which shows a wide range of variations ranging from 2.8 - 7.7 mg/g found in Romanian red. Allicin is very unstable and breaks immediately into nearly 200 other sulphur compounds, the reason being its high reactivity may cause damage to the plant itself (Josling, 2008).

Allicin is a miraculous bioactive compound produced from garlic that makes the herb an effective antibacterial, antifungal, antiparasitic, antiviral, antihypertensive, antiatherosclerotic agent (Ankri *et al*., 1999).

2.7.1 Structure of Allicin

Chemically allicin is Diallyl thiosulphinate, allyl sulphide or S-(2 Propenyl)2 propene-1-sulphinothioate which has a reactive part consisting of sulphur-sulphur bond attached to an oxygen atom. Chemists know that this combination is very reactive and consider it as the main reason for the antibiotic properties of allicin.

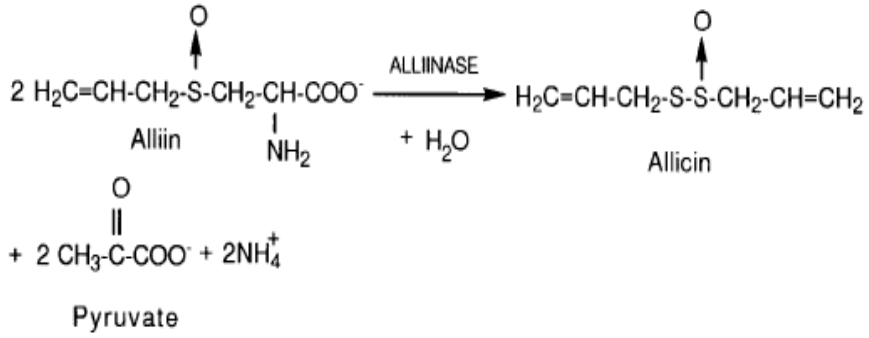


Figure 1. Generation of allicin in a garlic clove.

Ankri *et al.*, 1999

Allicin is considered as the main bioactive molecule of garlic which is released immediately after damage from which many daughter compounds are released that may or may not have a beneficial effect.

2.7.2 Mode of action of Allicin

Allicin is very keen to react with pathogens, it is able to penetrate the cell wall of the pathogen and interfere with its biochemical balance thereby hampering its activity. Under low concetrations, allicin damages the virulence of the pathogen without killing it but higher concentrations are fatal to the pathogens (Josling, 2008).

Microorganisms possess thiol - containing enzymes which are inhibited by the reaction of thiosulfinates with thiol groups thereby providing antibiotic activity to allicin

(Cavalitto and Bailey, 1944). Other bacterial enzymes such as acetyl- CoA- forming system, consisting of acetate kinase and phosphotransacetyl- CoA synthetase is also inhibited by allicin which is generally a noncovalent and reversible inhibition (Focke *et al.*, 1990).

Allicin is observed to strongly inhibit the cysteine proteinases, alcohol dehydrogenases and thioredoxin reductases essential for maintaining correct redox state in amoeba at a concentration as low as 10µg/mL (Ankri *et al.,* 1997).

In *Salmonella typhimuriumi* it was found that allicin causes bacteriostatic effect by partially inhibiting the DNA and protein synthesis with immediate effect being on RNA synthesis showing that it may be the primary target for Allicin (Feldberg *et al*., 1988). Studies show that allicin may react with the single sulfhydryl group present in the alpha subunit of RNA polymerase of *Escherechia coli* (Ozolin, 1989).

Despite of having a varied mode of action on different microorganisms, much work on the use of allicin as a pharmaceautical molecule has not been done.

2.7.3 Allicin and garlic extracts against fish ectoparasites

In a study by Chitmanat *et al.* (2004) it was found that the crude extracts of both Garlic and Indian almond were able to eliminate *Trichodina* infection from tilapia at a concentration of 800mg/L.

Gholipour *et al.* (2012) found in a study that *Ichthyophthirius mutifiliis* infected molly (*Poecilia latipinna*) was treated in 5 days when given a bath treatment with 0.1g/L garlic extract.

Schelkle *et al.* (2013) tested the effect of different garlic preparations against *Gyrodactylus turnbulli* infecting guppy and found that Chinese freeze dried garlic was equally effective as levamisole and can be used as an alternative in controlling gyrodactylids in fish.

Militz *et al.* (2013) reported that when *Lates calcarifer* was fed with feed incorporated with garlic extract @ 50-150ml/Kg, the infection rate by the oncomiracidia of *Neobenedenia sp.* reduced to less than 10 % concluding that garlic extract can be used for controlling *Neobenedenia sp.* in marine culure system.

Fridman *et al*. (2014) found that when *Gyrodactylus turnbulli* infected guppies were given a bath treatment in aqueous garlic extract at 7.5ml/L and 12.5ml/L, the population of the parasite reduced significantly. Similar results were observed when infected fishes were fed with garlic extract incorporated feed at 10% and 20 % concentration for 14 days.

Alnassan *et al.* (2015) reportd that allicin at concentration 1.8mg/L was effective against *Eimeria tenella* sporozoites which causes coccidiosis in chicken eggs.

2.7.4 Acute toxicity of allicin and garlic extracts in fish

It was found in a study that the LC_{50} (2 hrs) of crude garlic extract in tilapia was 2,259.44 mg/L and exposed fishes showed rapid opercula movement and erratic swimming beahaviours (Chitmanat *et al.,* 2004).

Syngai *et al*. (2016) reported that the LC_{50} value for Aqueous extract of garlic for *Cyprinus carpio* was 253.19 mg/L indicating that it is safe for this species of fish and the reason behind fish mortalities was found to be a decrease in pH value.

Fridman *et. al.* (2014) reported that guppies were able to tolerate garlic based treatment at 12.5ml/ L concentration.

2.8 Methanol and its toxicity to aquatic life

Methanol is an age old industrial chemical used since 1800s. Methanol consists of a single carbon atom, making it the simplest alcohol. It is colorless, volatile with a slight odour of alcohol and is completely miscible in water. It is widely used as a solvent in paint strippers, paints, carburetor cleaners etc. and also as a feed stock in place of other organic chemicals such as formaldehyde, acetic acid, chloromethane, and methyl

tert-butyl ether (MTBE). Compared to the release of gasoline and diesel in the environment, methanol is considered as safer when compared to them (Pirnie, 1999).

Kaviraj *et al.* (2004) reported that the 96 hrs LC_{50} of methanol for a cladoceran crustacea *Moina micrura* was 4.82g/L. In the same study it was reported that the 96 hrs LC_{50} of methanol for *Oreochromis mossambicus* was 15.2 g/L and for oligochaete worm *Branchiura sowerbyi,* it was found to be 54.89g/L.

According to the IPCS *i.e.* International Programme on Chemical Safety (WHO, 1997), the 48 and 96 hrs LC_{50} tests shows a tolerance of (1300 and 15900 mg/L respectively) in case of invertebrates and the 96h LC_{50} for fish was in the range 13000-29000 mg/L thereby considering methanol as less toxic to aquatic organisms.

Methanol is less toxic to aquatic life as because it is readily miscible in water and being a volatile substance, evaporates quickly in the atmosphere thereby reaching biodegradable levels very easily (Pirnie, 1999).

3. MATERIALS AND METHODS

3.1 Site of the Experiment

The Experiment was conducted in Central Wet Laboratory and AEHMD laboratory, ICAR-Central Institute of Fisheries Education, Mumbai, Maharashtra, India.

3.2 Selection of Experimental Fish

Ornamental fish used for the experiment was selected after conducting a pilot survey for the presence of ectoparasites. Three different species of fish viz. goldfish (*Carassius auratus*), koi carp (*Cyprinus carpio* Koi) and molly (*Poecilia sphenops*) were procured from Kurla, Borivali and Versova Aquarium shop, Mumbai , Maharashtra, India during the month of November in three consecutive weeks and were checked for the presence of ectoparasites such as *Argulus*, *Lernaea*, Monogenea, *Trichodina* and *Ichthyophthirius spp*. Following the sampling method given by (Bondad-Reantaso, 2001) in FAO, sixty fishes from each location were sampled and 20 fish of each species were considered as the minimum number of fish sample for screening purpose. Based on the prevalence and intensity of parasite infestations on the fish, it was found that goldfish was the most infected and hence it was chosen as the experimental animal. A total of 180 fishes were sampled for the presence of ectoparasites and their prevalence is given in (Table 2).

Healthy goldfish of average weight 4.5 ± 0.5 gms were procured from Kurla fish market in the month of December. The fishes were bought in polythene packing containing (50 fish/ Pack) with sufficient oxygen. On arrival, the fishes were transferred carefully into 500L capacity circular FRP tanks labeled (Tank 1), kept in the wet laboratory and were left undisturbed with sufficient aeration for the whole night. In order to compensate the handling stress, a mild salt treatment followed by a dip in 20ppm potassium permanganate solution was given to the fishes. The fishes were then left undisturbed with sufficient aeration for acclimatization for 15 days and were fed with 1 % bodyweight commercial goldfish pellet feed during the period.

3.3 Experimental Procedures

3.3.1 Sampling of fish for isolation of ectoparasites

150 goldfish of average weight 4.75±0.25 gms were procured from Kurla fish market, Mumbai, Maharashtra, India in the month of January and March. The fishes were bought in polythene bags containing 50 fish/ pack with sufficient oxygen. On reaching the wet laboratory, the fishes were immediately transferred into 500L circular FRP tanks and then each fish was examined for the presence of ectoparasites and segregated there on.

Argulus infested fish were identified by observing the fish by keeping in a 100ml capacity petriplate containing water and watching for the parasite on the skin, fins, belly regions and opercula whereas *Ichthyophthirius multifiliis* infested goldfish were identified by observing the presence of white spots on the body of fish and confirmation was done by observing a skin scrape from the infected fish under a microscope at 10X and 40X magnification. The remaining fish were observed for the presence of *Dactylogyrus* sp. on the gills by observing gill clips from 10 fishes.

Each fish was examined for the presence of different ectoparasites and in case of heavy mixed infection, the fishes were kept in a separate tank.

The fishes after being segregated by the presence of different parasites were kept in four different aquaria of 150 L capacity with continuous aeration and were fed with commercial goldfish pellet feed; the healthy fishes were kept with the previous batch of fishes after a salt treatment, followed by 20ppm potassium permanganate treatment in 500 L circular FRP tank.

3.3.2 Artificial infection of *Argulus spp* in goldfish

The *Argulus* infested fishes (segregated above in the sampling) were used for artificial infestation of healthy fishes. Two methods were used to conduct the artificial infection:

i) The first method was the cohabitation method (Saurabh *et al.,* 2010) wherein both heavily and moderately pre-observed *Argulus* infected fish were kept with healthy fish together in a ratio of 1:5 in the same aquaria. The *Argulus* infested fish were marked by making a small cut on the caudal fin so as to check the spread of infection. The fishes were observed every day, after one week for the increase in the number of parasites and spreading of *Argulus*.

ii) The second method was a modified form of (Kumar *et al.*, 2012b) where the artificial infection was carried out by first placing substrates like stone slab, plastic aeration pipes, macrophytes (*Hydrilla*), bamboo sticks and stones in a tank and *Argulus* infested fish were introduced in the same tank. Post spawning, *Argulus* had layed sufficient eggs on the substrates which were then transferred into different tanks containing healthy goldfish.

All the tanks used for artificial infection of *Argulus* was labeled as (Tank 2).

3.3.3 Artificial infection of *Ichthyophthirius multifiliis* in Goldfish

To get sufficient theronts for experiment, a trial for artificial infection of *I. multifiliis* with goldfish was performed by the following method.

Cohabitation method of artificial infection of *I. multifiliis* was performed as per (Saurabh *et al.,* 2010), wherein healthy goldfish were kept with ich infected fishes in the same tank. The fishes were observed every day for the increase in the number of parasite and spread of infection to other fishes. In case of fish mortality, the skin scraping containing the parasite were collected and released back into the same aquarium (Tank 3) and healthy fishes were added in the tank.

3.3.4 Cleaning and Siphoning

Cleaning of the tanks was done manually before putting up any experiment. Before releasing any new fish in the tank, the water in the tank was filled 24 hours prior to arrival of fish and was provided with continuous aeration. Siphoning was done from

the tank containing healthy fishes every day to remove the settled feed pellets and fecal matter and was replaced with an equal volume of bore well water at same temperature.

Siphoning from tanks containing *Argulus* infected fishes was done weekly or fortnightly. Here, the siphoned water containing feed pellets and fecal matter was transferred in a tub and was replaced with equal volume of bore well water. 2-3 healthy goldfish was kept in the siphoned water present in the tub for collecting any *Argulus* that might have been siphoned along with the water and were then transferred back into the tank containing *Argulus* infected fishes. If there was any decrease in the number of *Argulus* on fishes following siphoning, the tanks were not siphoned until the infection was restored.

No water exchange was carried out while performing the Acute toxicity test.

3.4 Physico- Chemical Parameters of Water

3.4.1 Temperature

The water temperature in each tank was meausured using digital thermometer make (ThermoScientific, USA)

3.4.2 pH

The pH of the water was measured using pH meter make (ThermoScientific, USA).

3.4.3 Dissolved Oxygen

The Dissolved Oxygen was estimated as per the protocol of (APHA, 2005) and calculated as follows :

$$\text{Dissolved Oxygen (mg/L)} = \frac{\text{Volume of Sodium thiosulphate x 0.2 x 1000}}{\text{Volume of Sample taken}}$$

3.4.4 Free Carbon Dioxide

The Dissolved free Carbon Dioxide was measured following the method of (APHA, 1998) and was calculated using the following formula:

$$CO2\ (mg/L) = \frac{A\ X\ N\ X\ 1000}{Volume\ of\ Sample\ taken}$$

Where, A= volume of titrate (NaOH), N= normality of titrate (N/44)

3.4.5 Total Alkalinity

The total alkalinity was measured according to (APHA, 2005) and was calculated as follows:

$$Total\ Alkalinity\ (mg/L) = \frac{No.\ of\ ml\ of\ H2SO4\ consumed\ X\ 1000}{Volume\ of\ Sample\ taken}$$

3.5 Herbal Extracts

Two herbal extracts (commercial grade) namely Azadirachtin (EC 21.5%) and Allicin (EC 100%) were procured from SOM Phytopharma, Hyderabad, India and Allimax, Chicago, USA respectively.

3.6 Glassware, Plastic ware and Chemicals

The Glassware used throughout the experiment was neutral glass of Borosil Make (Mumbai). The plastic ware was from Tarsons Pvt Ltd (Mumbai). Chemicals used during the experiment were mainly from Hi-media (Mumbai), Qualigen (Mumbai), Merck (Germany), SRL (Mumbai), Sigma (Mumbai) etc.

3.7 Experimental Design

3.7.1 Antiparasitic effect of azadirachtin (EC 21.5%) against common ectoparasites of goldfish.

3.7.1.1 Preparation of azadirachtin stock solution and working test solution

Azadirachtin has a high affinity towards polar organic solvents and is easily soluble in them (Morgan *et al.,* 1968). Stock solution was prepared as per technical details of AzapureTM (Vittal Mallya Scientific Research Foundation). The stock solution of concentration 1000ppm was prepared by dissolving 1g of azadirachtin (EC 21.5%) in 5 ml of methanol and finally making the total volume to 215 ml by addition of distilled water. The working test solutions of different concentrations for test against different parasites were made using this stock solution. The stock solution and working solutions were prepared freshly as on the day *in vitro* test was performed.

3.7.1.2 Preparation of stock solution of diluent control (2.32% methanol).

Since Azadirachtin was being dissolved in methanol prior to diluting in distilled water, a positive control of the same amount of methanol but without azadirachtin was made as follows. 5 ml of methanol in 210 ml of distilled water and without azadirachtin was used as 2.32 % stock solution of methanol. Each time this positive control was prepared freshly and was used at the same concentration as the highest dose of treatment given from Azadirachtin stock solution.

3.7.1.3 In vitro antiparasitic efficacy of azadirachtin against *Argulus spp*

3.7.1.3.1 Collection of adult and juvenile Argulus from infected fish

The *Argulus spp* were collected from the infected fish by a modified method of (Kumar *et al.,* 2012b). The heavily infected goldfish were kept in a 100 ml capacity petri plate containing filtered aquarium water and was shaken gently. Some of the attached *Argulus* from the fish were released by its jerking movement. The remaining *Argulus* were picked carefully from the fish with the help of fine forceps and placed in the same

petri plate containing *Argulus*. From here, actively swimming parasites were considered for performing the *in vitro* assay to evaluate the antiparasitic efficacy of Aza.

3.7.1.3.2 Definitive range finding study of azadirachtin for conducting *in vitro* bioassay test against *Argulus*

For determining the final concentrations of azadirachtin to be used for the *in vitro* assay against *Argulus*, a pilot study was conducted with concentrations 1, 5, 10, 15, 20 mg L^{-1}; 30, 60, 90 and 120 mg L-1; 50, 100, 150, 200 mg L^{-1}. Six parasites were used for each treatment given in duplicate and were observed continuously, every 30 minutes for their mortality. The time taken for parasite mortality in these concentrations was noted down along with the time of maximum survivability of parasites in both diluent (2.32% methanol devoid of Aza) and negative control (filtered aquarium water). Correlating the obtained results, following definitive range of Azadirachtin was used against *Argulus* for *in vitro* bioassay to evaluate the antiparasitic efficacy of Aza; T_1 (25 mg L^{-1}), T_2 (50 mg L^{-1}), T_3 (75 mg L^{-1}), T_4 (100 mg L^{-1}) and T_5 (125 mg L^{-1}).

3.7.1.3.3 Definitive in *vitro* test of azadirachtin against juvenile and adult Argulus

18 live actively swimming parasites were subjected to 50 ml of five different concentrations of azadirachtin test solutions viz. T_1 (25 mg L^{-1}), T_2 (50 mg L^{-1}), T_3 (75 mg L^{-1}), T_4 (100 mg L^{-1}) and T_5 (125 mg L^{-1}). At every 30 minutes the parasites were observed for their mortality until all parasites had died in the lowest concentration. The parasite was considered as dead when they did not exhibit any movement after observing for 5 minutes and then by touching slightly with a feather forceps. The treatments were given in triplicate and was compared with a diluent control group containing 2.32 % methanol but no azadirachtin.

3.7.1.3.4 Effect of azadirachtin on *Argulus* eggs under *in vitro* conditions

Argulus eggs from the same tank were collected from tank 2 containing artificially infected goldfish with *Argulus*. The confirmation of the eggs belonging to the same stages was done by observing them under a microscope for presence of eye spots, and crudely by noticing their colour. Since, every day eggs were observed in the tanks, it

made easier to choose eggs of same developmental stage. Post observation, clutch was divided in such a way that each subsection consisted of 30 eggs each.

Thirty eggs were exposed to 50 ml solution of the following concentrations of azadirachtin; T_1 (25 mg L^{-1}), T_2 (50 mg L^{-1}), T_3 (75 mg L^{-1}), T_4 (100 mg L^{-1}), T_5 (125 mg L^{-1}), T_6 (150 mg L^{-1}) and T_7 (175 mg L^{-1}) in petri plates. The petri plates were maintained in natural photoperiod (12:12h) of light and darkness for the development and hatching of eggs. The temperature during the experimental period was 29.5 – 31.5^0 C. The eggs were observed at every 12h until 100% hatching had occurred in the control groups. The solution in the treatment and control groups were refilled regularly. All treatments were provided 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.

3.7.1.4 I*n vitro* antiparasitic efficacy of azadirachtin against *Ichthyophthirius multifiliis* theronts

3.7.1.4.1 Collection of Theronts for experiment

The *I. multifiliis* theronts were collected using a method as per (Clayton and Price, 1988). The heavily infested goldfish with *I. multifiliis* obtained from the aquarium shop previously were placed in several 100ml capacity petri plates containing filtered aquarium water for 30 mins. Mature parasites were freely dislodged from the fish by its body movement. Actively moving parasites were transferred to a fresh petri plate containing filtered tank water with the help of a Pasteur pipette. These parasites were incubated at 23±0.50 °C for 20-24 h for getting the theront stage.

3.7.1.4.2 Definitive range finding study of azadirachtin for conducting *in vitro* bioassay test against *Ichthyophthirius multifiliis* theronts

In order to find a suitable range of concentrations of azadirachtin to be used against *I. multifiliis* theronts, a pilot study was conducted *in vitro* with different concentrations of azadirachtin. Theront suspension containing 300 theronts were

subjected to 1(0.95), 5(4.76), 10(9.52), 15(14.28), 20(19.05) mg L^{-1}; 40(38.09), 60(57.14), 80(76.19), 100(95.23) mg L^{-1} of azadirachtin solutions in duplicates. The time taken for mortality of theronts in treatment groups and the maximum time of survival of theronts in control groups were noted down. Effective concentration against the theronts within 3-6 hours' time were selected and a final range of concentrations of azadirachtin was decided as T_1 10(9.52 mg L^{-1}), T_2 20(19.05 mg L^{-1}), T_3 30(28.57 mg L^{-1}), T_4 40(38.09 mg L^{-1}) and T_5 50(47.61 mg L^{-1}) for the definitive *in vitro* assay.

3.7.1.4.3 Definitive *in vitro* test of azadirachtin against *Ichthyophthirius multifiliis* theronts

The concentration of theronts was estimated as per (Straus and Meinelt, 2009) by placing 10 droplets of 2 µl each on a glass slide and observing the organism under microscope at 10 X and 40 X magnification. The mean number of theronts in each droplet was extrapolated to estimate the final concentration. A minimum of 300 theronts was used for *in vitro* assay. 200 µl of working test solution was poured in each well of 96 well plate and 300 theronts were added to each treatment to get a final concentration of T_1 10(9.52 mg L^{-1}), T_2 20(19.05 mg L^{-1}), T_3 30(28.57 mg L^{-1}), T_4 40(38.09 mg L^{-1}) and T_5 50(47.61 mg L^{-1}). Post exposure, the theronts were continuously observed for its mortality until all parasites had died in the lowest concentration. Theronts with abnormal morphology and lack of motility were considered as dead. The Treatments were given in triplicate and was compared with a positive control group containing 2.32 % methanol without azadirachtin.

3.7.1.5 I*n vitro* antiparasitic efficacy of azadirachtin against *Dactylogyrus spp*

3.7.1.5.1 Collection of *Dactylogyrus spp* for experiment

Small gill clip containing 3-6 filaments were cut out from the infected fish procured from the market after anaesthetizing it with 50 µl/L clove oil (Merck, Germany) and were observed under the microscope at 10 X for the presence and intensity of parasite by placing the gill clip on a cavity slide containing filtered tank water. Once the presence of parasite was confirmed, the *in vitro* test was conducted.

3.7.1.5.2 Definitive range finding study of azadirachtin for conducting *in vitro* bioassay test against *Dactylogyrus spp*

For determining the dose of different treatments to be used for the *in vitro* bioassay of azadirachtin against *Dactylogyrus spp,* a pilot study consisting of the following concentrations viz. 25, 50, 75, 100 mg L^{-1}; 15, 30, 45, 60, 90 mg L^{-1} in duplicate was performed prior to the final test. 20 parasites were subjected to the mentioned concentrations of azadirachtin and were observed regularly for their mortality.The time of parasite mortality and maximum time of survivability in treatment and control groups respectively was noted down. Based on the result of pilot study, the doses that were able to kill the parasite in 3-4h were chosen as base for determining the final concentrations of azadirachtin to be used in the *in vitro* test.

Seven treatments of azadirachtin T_1 (1 mg L^{-1}), T_2 (5 mg L^{-1}), T_3 (10 mg L^{-1}), T_4 (15 mg L^{-1}), T_5 (20 mg L^{-1}), T_6 (25 mg L^{-1}) and T_7 (30 mg L^{-1}) concentrations were used in the definitive *in vitro* test.

3.7.1.5.3 Definitive *in vitro* test of azadirachtin against *Dactylogyrus spp*

The *in vitro* test was conducted as per (Fridman *et al*., 2014). Gill clips containing 35-40 parasites were transferred in a well of 96 well microtitre plate containing fitered tank water. They were then observed under a dissecting microscope to check whether the transfer was successful or not. At this moment, any dead or mouribund parasite was removed using a micropipette and 30 active parasites attached to the gill were transferred into 200µl test solution of T_1 (1 mg L^{-1}), T_2 (5 mg L^{-1}), T_3 (10 mg L^{-1}), T_4 (15 mg L^{-1}), T_5 (20 mg L^{-1}), T_6 (25 mg L^{-1}) and T_7 (30 mg L^{-1}). The parasites were continuously observed under the Dissecting microscope at regular intervals of 30 minutes. Parasites with lack of motility even after giving a gentle stream of water current with a fine needle and showing abnormal morphology were considered as dead. A diluent control containing 2.32% methanol and a negative control containing filtered aquarium water was retained along with treatment groups for comparison.

3.7.1.6 Acute toxicity of azadirachtin for goldfish.

In order to find out the acute toxicity of azadirachtin (EC 21.5%) on goldfish, a setup to find the 96 hours median lethal concentration (LC_{50}) of Aza against goldfish was arranged in wet laboratory. A static short term toxicity test was conducted according to (APHA, 2005). To determine the LC_{50}, range finding to exposure of azadirachtin was done using following concentrations; 0, 20, 40, 60, 80, 100, and 120 mg L^{-1}; and finally the definitive test was conducted in two sets with 1, 2.5, 5, 7.5 and 10 mg L^{-1}concentrations of azadirachtin for 96h and 10, 20, 30, 40 and 50 mg L^{-1} for 24h along with a diluent control of 2.32 % methanol without azadirachtin and a negative control containing fishes in filtered bore well water.

Briefly, the procedure consisted of 6 goldfish of average weight (3.25 ± 0.25 gms) kept in 30 litres of the test solutions and a control in aquarium. Continuous aeration was provided during the test period. The fishes were not fed throughout the test period. The fishes were observed at every 3 hours interval upto 24h for any mortality of fish, then every 6 h upto 48 h and then every 24 h until the completion of the test. Death was considered when the fishes lacked mobility even after touching with a glass rod. Any dead fishes were immediately removed from the aquarium during the course of the test period.

3.7.2 Antiparasitic effect of allicin (EC 100%) against common ectoparasite(s) of Goldfish.

3.7.2.1 Preparation of Allicin Stock Solution and working solutions:

Available allicin was readily soluble in water. Stock solution of 10 mg ml^{-1} of allicin was made by dissolving 10g of allicin powder in 1L of Distilled water. From this stock solution, different working test solution was made as per the requirement of treatments.

3.7.2.2 *In vitro* antiparasitic efficacy of allicin against *Argulus spp*

3.7.2.2.1 Collection of *Argulus* from infected fish

The *Argulus* from the infected fishes were collected in petriplates as mentioned above (3.7.1.3.1).

3.7.2.2.2 Definitive range finding study of allicin for conducting *in vitro* bioassay test against *Argulus*

To determine the final concentrations of allicin to be used for the *in vitro* assay, a pilot study of allicin against *Argulus* was conducted using concentrations such as 0.025, 0.05, 0.075 and 0.1 mg ml^{-1}; 0.2, 0.4, 0.6, 0.8 and 1 mg ml^{-1}; 2, 4, 6 and 8 mg ml^{-1}. Six parasites were subjected to 50 ml of mentioned treatments in duplicates. Based on the time of mortality of parasites in treatment groups and maximum time of survival in control, the final concentrations of allicin used for *in vitro* assay against Argulus was decided as; T_1 (2 mg ml^{-1}), T_2 (2.5 mg ml^{-1}), T_3 (3 mg ml^{-1}), T_4 (3.5 mg ml^{-1}) and T_5 (4 mg ml^{-1}).

3.7.2.2.3 Definitive *in vitro* test of allicin against *Argulus*

Eighteen actively swimming parasites were chosen for the *in vitro* assay. They were subjected to 50ml of five different concentrations of allicin; T_1 (2 mg ml^{-1}), T_2 (2.5 mg ml^{-1}), T_3 (3 mg ml^{-1}), T_4 (3.5 mg ml^{-1}) and T_5 (4 mg ml^{-1}). The parasites were observed every 30 minutes for their mortality which was considered when the parasite showed no movements for 5 minutes after continuous observation and confirmation was done by touching the parasite with a hairbrush for 5 minutes. All treatments were provided in triplicates along with a control containing filtered aquarium water.

3.7.2.3 *In vitro* antiparasitic efficacy of allicin against *Dactylogyrus spp*

3.7.2.3.1 Collection of *Dactylogyrus spp.* from infected fish

The parasite was collected in the same way as mentioned in the previous section (3.7.1.5.1).

3.7.2.3.2 Definitive range finding study of allicin for conducting *in vitro* bioassay test against *Dactylogyrus spp*

A pilot study to check the efficacy of Allicin against *Dactylogyrus spp.*was performed in order to decide the treatments to be given in the final *in vitro* assay. 20 actively moving parasites attached to gill clips were transferred carefully into well of 96 well microtitre plate containing 200 µl of Allicin solution of the following concentrations; 0.025, 0.05, 0.075 and 0.1 mg ml^{-1}; 0.2, 0.4, 0.6, 0.8 and 1 mg ml^{-1}; 2, 4, 6, 8 mg $ml^{-1}$and 10 mg ml^{-1}. Based on the survivability of parasite in the control group and time of mortality in treatment groups, the following concentrations of allicin was used in the definitive *in vitro* test; T_1 (1 mg ml^{-1}), T_2 (1.5 mg ml^{-1}), T_3 (2 mg ml^{-1}), T_4 (2.5 mg ml^{-1}), T_5 (3 mg ml^{-1}), T_6 (3.5 mg ml^{-1}) and T_7 (4 mg ml^{-1}).

Concentrations of allicin that were effective in killing the parasites in 3-4 h were used as baseline for deciding the final dose.

3.7.2.3.3 Definitive *in vitro* test of allicin against *Dactylogyrus spp*

The test was performed as per (Fridman *et al.,* 2014). A gill clip was cut out from the infected goldfish and was observed in a cavity slide containing water under a microscope at 10 X power. In case of sufficient presence of parasite, the gill clip was transferred to a well of 96- well micro titre plate containing filtered aquarium water and was checked under a dissecting microscope, to ensure that the transfer was successful. From here, 30 actively moving parasites attached to the gill clips were exposed to 200µl of following concentrations of allicin; T_1 (1 mg ml^{-1}), T_2 (1.5 mg ml^{-1}), T_3 (2 mg ml^{-1}), T_4 (2.5 mg ml^{-1}), T_5 (3 mg ml^{-1}), T_6 (3.5 mg ml^{-1}) and T_7 (4 mg ml^{-1}). The parasites were observed continuously for their mortality which was considered on the basis of absent of movement and abnormal morphology of the parasite and was compared with a control group containing filtered aquarium water with the parasites.

3.7.2.3.4 Acute toxicity of Allicin against Goldfish.

In order to find out the acute toxicity of allicin (EC 100%) on goldfish, a setup to find the 96 hours median lethal concentration (LC_{50}) of allicin for goldfish was arranged

in wet laboratory. A static short term toxicity test was conducted according to (APHA, 2005). To determine the LC_{50}, range finding to exposure of allicin was done using following concentrations; 0, 0.2, 0.4, 0.6, 0.8, 1, 1.5 and 2 mg ml $^{-1}$ and finally the definitive test was conducted with 0, 1, 1.2, 1.4, 1.6, 1.8 and 2 mg ml^{-1}concentrations of allicin for 96 hours along with a negative control containing fishes in filtered bore well water.

Briefly, the procedure consisted of 6 goldfish of average weight (3.25 ± 0.25 gms) kept in 30 litres of the test solutions and a control in aquarium. Continuous aeration was provided during the test period. The fishes were not fed throughout the test period. The fishes were observed at every 3h interval upto 24 h for any mortality of fish, then every 6h upto 48h and then every 24h until the completion of the test. Death was considered when the fishes lacked mobility even after touching with a glass rod. Any dead fishes were immediately removed from the aquarium during the course of the test period.

3.8 Compilation and Analysis of Data

3.8.1 Prevalence of etoparasites on sampled ornamental fish

Prevalence of ectoparasites was calculated using Microsoft excel sheet 2010 using the following formula:

$$\text{Prevalence (\%)} = \frac{\text{No. of infected fish}}{\text{Total number of fish sampled}}$$

3.8.2 Determination of median effective concentration (EC_{50}) of azadirachtin and allicin against common ectoparasites infesting goldfish

A mortality chart of parasite was made with respect to time and associated concentration of the respective HBMs. The median effective concentration (EC_{50}) of azadirachtin and allicin against individual parasite was determined by analysing the data through probit analysis using SPSS 16.0 version at 99% confidence level.

Tables and charts for EC_{50} of selected HBMs against ectoparasites were prepared using Microsoft excel sheet 2010.

3.8.3 Calculation of *in vitro* Antiparasitic efficacy (A.E.) of azadirachtin and allicin against different ectoparasites of goldfish

The *in vitro* antiparasitic efficacy of both the herbal bioactive molecules was calculated using the equation (Kumar *et al.,* 2012)

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

Where, AE = antiparasitic efficacy of HBMs against different ectoparasites, B is the mean number of surviving parasites in the positive control and T is the mean number of surviving parasites in treatment.

3.8.4 Determination of median lethal concentration (LC_{50}) of allicin and azadirachtin for goldfish

A mortality chart of fish at different effective concentrations of HBMs with respect to time was prepared. The LC_{50} was then determined by analysing this data through probit analysis using SPSS 16.0 version at 99% confidence level.

A chart of LC_{50} of both HBMs for fish against time was plotted using Microsoft excel sheet 2010.

3.8.5 Estimation of Therapeautic index

The therapeautic index of azadirachtin and allicin was estimated for selected ectoparasites using the formula:

$$\text{Therapeautic index} = \frac{\text{LC50 of the host}}{\text{EC50 against ectoparasites}}$$

A chart was prepared for therapeautic index of both allicin and azadirachtin against common ectoparasites of goldfish using Microsoft excel sheet 2010.

4. RESULTS

4.1 Prevalence of ectoparasites on sampled Ornamental fish

A total of 180 ornamental fish (goldfish, molly and koi carp) were sampled from three locations in Mumbai viz. Kurla, Borivali and Versova for the presence of ectoparasites. It was found that 32.77% of the fish were infected with different ectoparasites (*Argulus, Dactylogyrus, Gyrodactylus Ichthyophthirius, Lernaea and Trichodina*), out of which goldfish was the most infected fish with 56.67% prevalence of ectoparasites followed by koi carp (23.33%) and molly (18.33%), (Table 3).

4.2 Physico- chemical parameters of water

The physico-chemical parameters of water such as temperature, pH, dissolved oxygen (mg L^{-1}), free carbon dioxide (mg L^{-1}) and alkalinity (mg L^{-1}) were recorded from tanks containing healthy goldfish and *Argulus* and *Ichthyophthirius multifiliis* infected goldfish and also from the treatment groups while performing acute toxicity test for goldfish. The average values of the water quality parameters are represented in range (min-max) in (Table 4).

4.2.1 Temperature

The temperature of water from tanks containing *I multifiliis* infected fish ranged from 23.5^0 C to 27.4^0 C, *Argulus* infected fish ranged from 23.5^0 C to 31.2^0 C and healthy fish ranged from 23.5^0 C to 30.2^0 C (Table 4). The temeperature of experimental tanks while performing acute toxicity test of HBMs on goldfish ranged from 29.4^0 C to 31.5^0 C.

4.2.2 pH

There was very little pH variation throughout the experimental period, with water from all tanks showing pH in the range of 7.3 to 8.1 (Table 4). The pH of water while performing acute toxicity test of HBMs on goldfish was in range 7.6 to 7.9.

Table 3: Prevalence of ectoparasites on three different fish species sampled from three locations from Mumbai, Maharashtra, India.

Sl No.	Fish species	Total number of fish	Total number of infected fish	Prevalence (%)
1	Goldfish	60	34	56.67
2	Koicarp	60	14	23.33
3	Molly	60	11	18.33
4	Total	180	59	32.77

4.2.3 Dissolved Oxygen

The dissolved oxygen concentration in water from all tanks was in the range of 6.4 to 7.4 mg L^{-1} (Table 4) and during acute toxicity test of HBMs on goldfish it was found in the range of 6.4 to 6.8 mg L^{-1}.

4.2.4 Free Carbon Dioxide

The water from experimental tanks during the experimental period showed negligible concentration of free carbon dioxide in them (Table 4).

4.2.5 Alkalinity

The alkalinity of water in experimental tanks was observed to be in the range of 182 – 208 mg L^{-1} (Table 4). While performing acute toxicity test, the alkalinity of water was in the range 164-185 mg L^{-1}

4.3 Artificial Infection of Goldfish with Common Ectoparasites

4.3.1 Artificial infection of goldfish with *Argulus* spp.

Prominent infestation of *Argulus* was observed on goldfish from third week after the setup of artificial infection challenge. Every fish was infected on an average with 40-50 juvenile parasites, showing signs such as haemorrhages on fins, slow body movement, reduced feeding and mortality in case of heavy infestation (Plate 1 and Plate 2). The intensity of *Argulus* on randomly selected ten fish after artificial challenge test is given in (Table 5).

The substrates used during the artificial infection with *Argulus* were checked for the presence of *Argulus* egg clutch and was found that aquarium glass surface and stone slab were the most preferred substrate followed by sticks, stones, aeration pipe and hydrilla for laying eggs. The preference was assumed based on the number of egg clutches and eggs per clutch with respect to the size of the substrate. The images of *Argulus* egg clutch on different substrates are presented in (Plate 3 and 4).

Table 4: Physico-chemical parameters of water during the artificial infection of goldfish with *Argulus* and *I. multifiliis*

Parameters	Tank 1	Tank 2	Tank 3
Temperature (^{0}C)	23.5-30.2	23.5-31.2	23.5-27.4
pH	7.6-8.1	7.3-7.9	7.4-8.0
Dissolved Oxygen (mg L^{-1})	6.6-7.4	6.5-7.2	6.4-7.1
Free Carbon dioxide (mg L^{-1})	ND	ND	ND
Alkalinity (mg L^{-1})	192-205	182-196	201-208

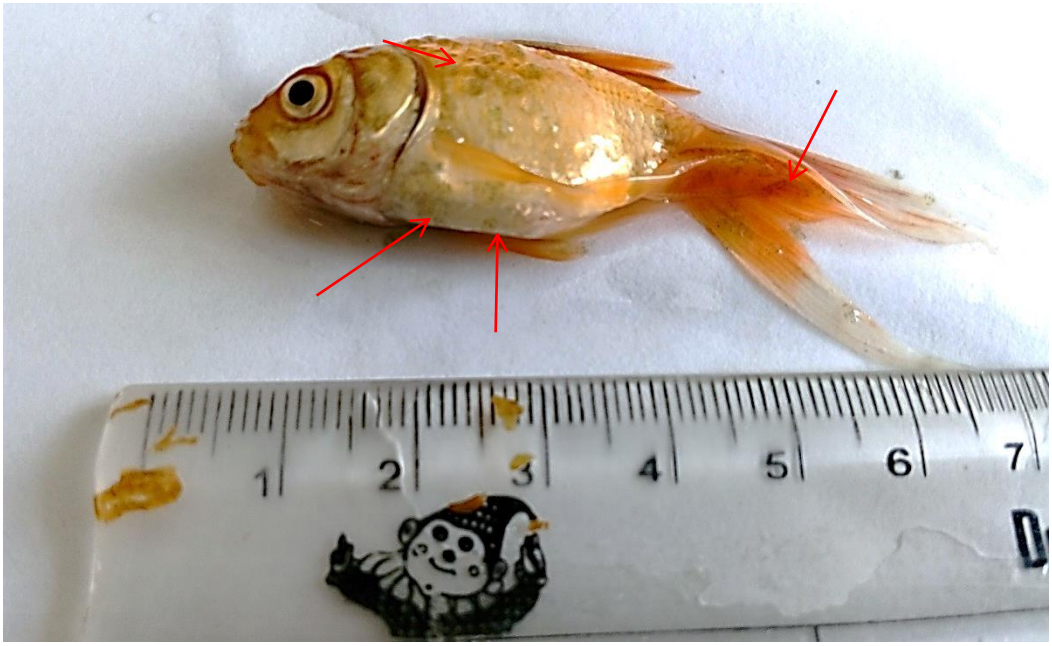
Tank 1- Containing healthy goldfish

Tank 2- Containing *Argulus* infected goldfish

Tank 3- Containing *I. multifiliis* infected goldfish

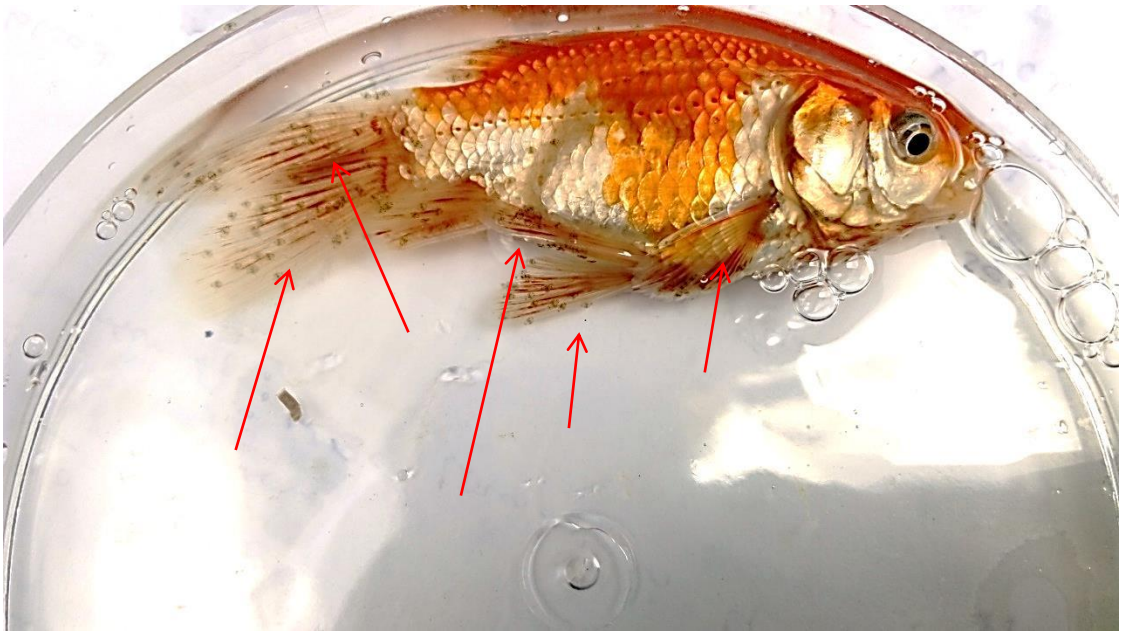
ND- Not Detectable

Hundreds of young *Argulus* attached to the body surface and caudal fin of goldfish



***Argulus* infection on belly regions, caudal fin, throat and body surface of goldfish**

Plate 1: Artificially infected goldfish with *Argulus*



Goldfish infected with *Argulus* showing haemorrhages in caudal fin, pectoral fin and pelvic fin.

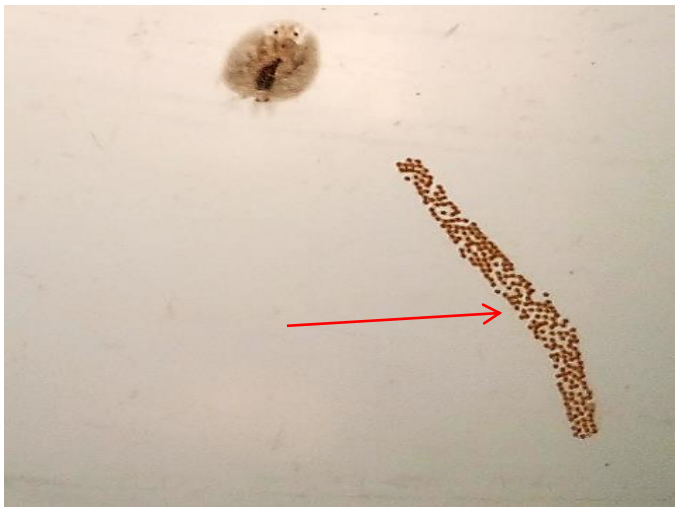
Goldfish infected with *Argulus* showing severe haemorrhages of caudal, pectoral, dorsal, anal and pelvic fin

Plate 2. Artificially infected goldfish with *Argulus* showing haemorrhages on fins

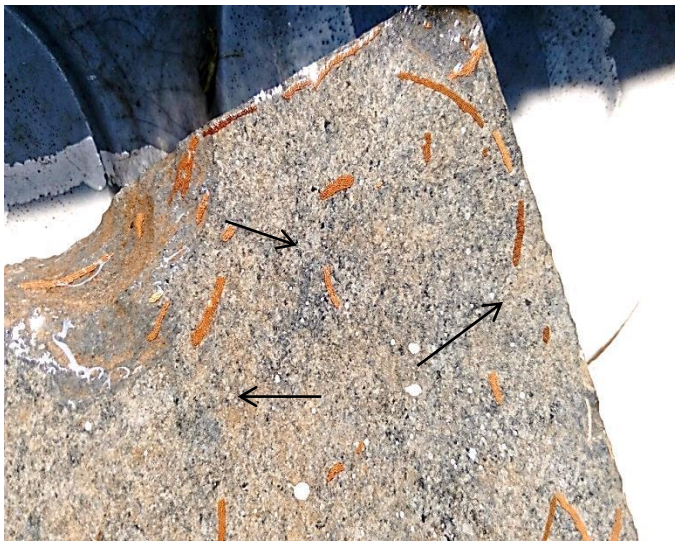
Table 5: Intensity of *Argulus* on goldfish during artificial infection challenge study

Sample No.	Prevalence	Intensity (No. of *Argulus*)
1	+	120
2	+	165
3	+	48
4	+	32
5	+	124
6	+	20
7	+	54
8	+	27
9	+	92
10	+	64

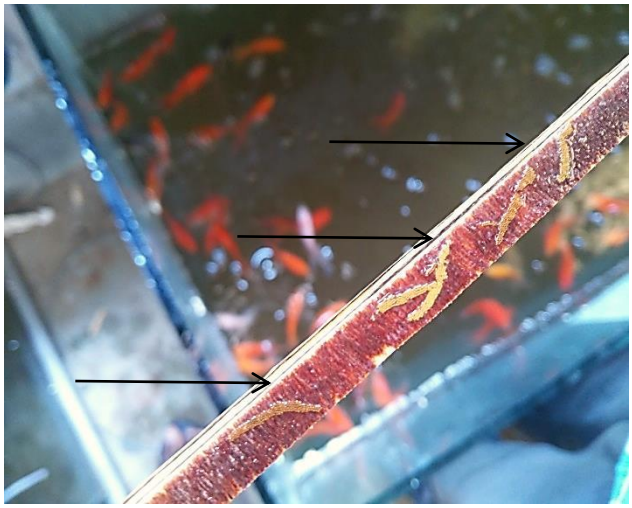
+ Positive sample (infested with *Argulus*)



***Argulus* egg clutch on glass**



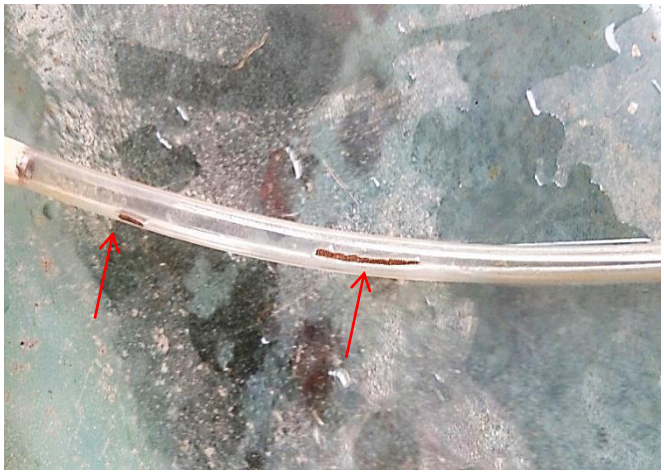
***Argulus* egg clutch on stone slab**



***Argulus* egg clutch on wooden stick**

Plate 3: *Argulus* egg clutch on different substrates

***Argulus* egg clutch on stone**



***Argulus* egg clutch on aeration pipe**

***Argulus* egg clutch on stem of hydrilla**

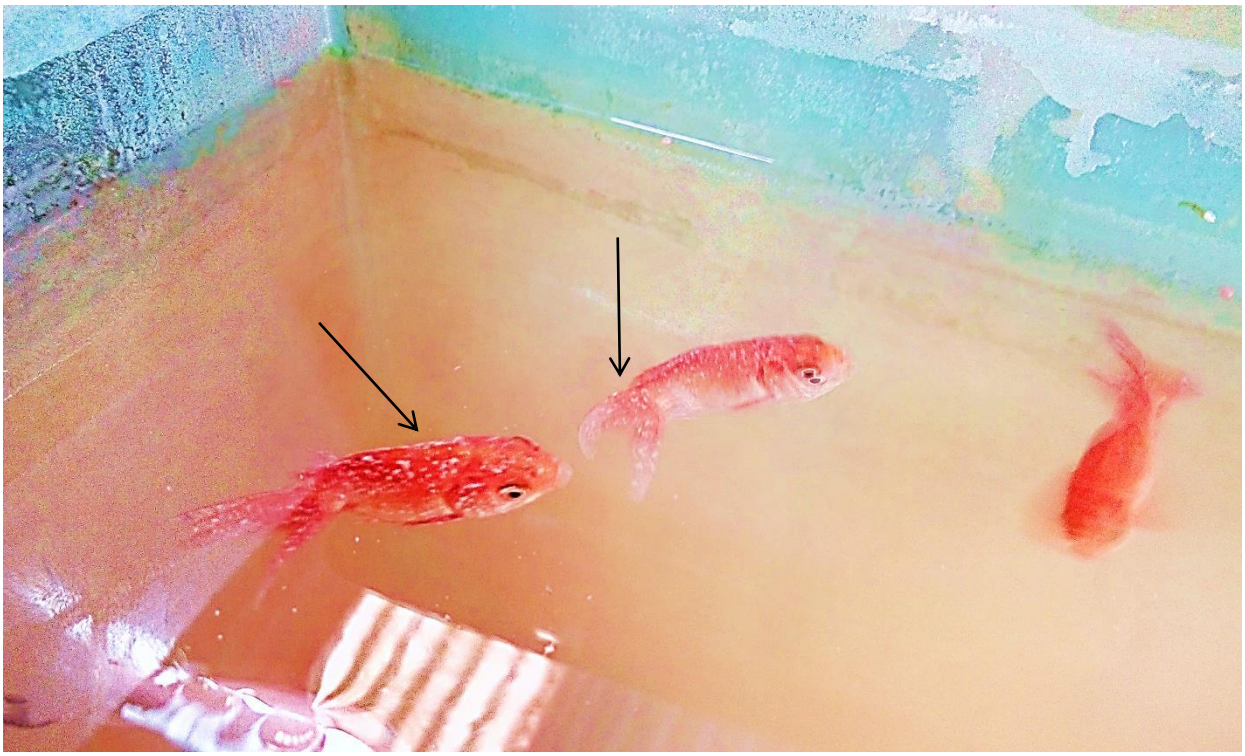
Plate 4: *Argulus* egg clutch on different substrates

The *Argulus* was mainly found attached on the caudal fin, body surface, belly and throat regions of the goldfish with adult *Argulus* preferably found on the caudal peduncle of the fish.

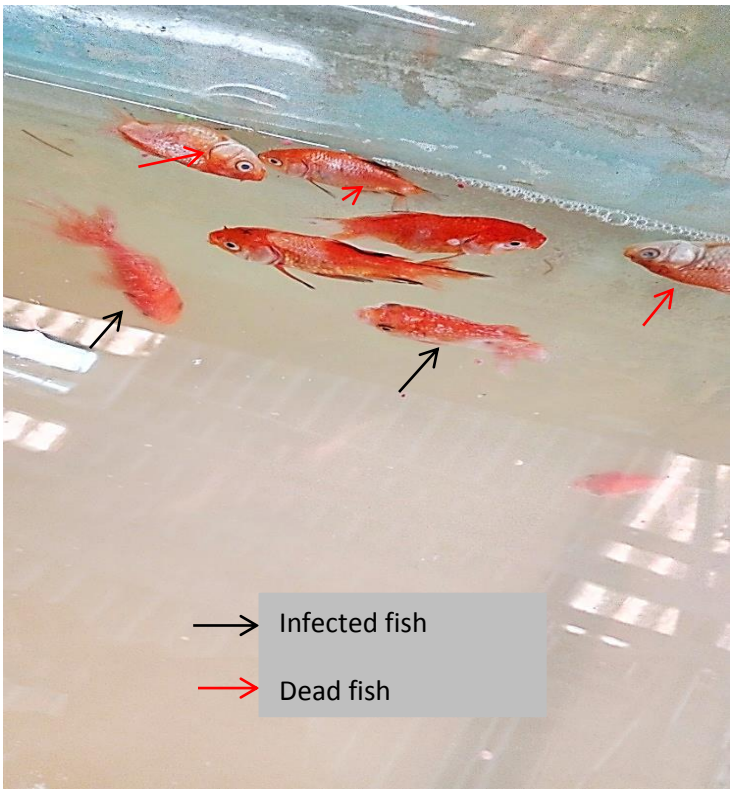
4.3.2 Artificial infection of goldfish with *Ichthyophthirius multifiliis*.

The healthy goldfish kept in cohabitation with the Ich infected goldfish showed prominent infection from the first week after the artificial challenge. Numerous white spots like crystals of salt granules were observed throughout the body of goldfish except on the belly regions (Plate 5). The major pathological signs observed were reduced feeding, surfacing, sluggish movement and mortality of infected fish. The parasites were mainly distributed in the caudal fin, dorsal fin, head and lateral surface of the fish body.

The dead fish were removed every day from the tanks and were substituted with an equal number of naive goldfish to maintain the *I. multifiliis* infection. Due to heavy mortality of fish and limitation of the parasite to survive at higher temperatures, a bulk infection of *I. multifiliis* could not be maintained (Plate 5).



Artificially infected goldfish with *I. multifiliis*



Mortality of goldfish due to heavy infection of *I. multifiliis.*

Plate 5: Artificially infected goldfish with *I. multifiliis* and associated mortality

4.4 *In vitro* study of Azadirachtin (EC 21.5%) and Allicin (EC 100%) against Common Ectoparasites Infesting Goldfish

4.4.1 Antiparasitic effect of azadirachtin against common ectoparasites of goldfish

4.4.1.1 *In vitro* antiparasitic effect of azadirachtin against adult *Argulus* spp.

In the present *in vitro* study, treatment of adult *Argulus* with different concentrations of azadirachtin showed a significant dose dependent mortality of the parasite when compared to control. The cumulative average and percentage mortality of *Argulus* at different time intervals was observed and recorded as shown in (Table 6). The cumulative percentage mortality is also shown in (Fig 1). The median effective concentration (EC_{50}) of azadirachtin against adult *Argulus* was calculated and represented in (Table 7) as well as in Fig 2. which shows that the 9, 12 and 15h EC_{50} of azadirachtin against *Argulus* was 61.29, 39.04 and 27.18 mg L^{-1} respectively. The *in vitro* study showed that the antiparasitic efficacy of azadirachtin test solutions viz T_1 (25 mg L^{-1}), T_2 (50 mg L^{-1}), T_3 (75 mg L^{-1}), T_4 (100 mg L^{-1}) and T_5 (125 mg L^{-1}) was 50%, 66.67%, 77.58%, 100% and 100% respectively in 12h and 61.11% and 83.33%, 100%, 100% and 100% respectively in 15h (Table 8) showing that azadirachtin possesses antiparasitic effect against adult *Argulus*. The morphology of the parasite before and after treatment was noted (Plate 6). Notably there was no mortality of *Argulus* encountered in the control group upto 36 h.

4.4.1.2 *In vitro* antiparasitic effect of azadirachtin against juvenile *Argulus* spp.

In the present *in vitro* study, a similar dose dependent mortality of juvenile *Argulus* was observed when compared to control. The cumulative average and percentage mortality of the parasite was observed and calculated as represented in Table 9. Fig 3. represents the cumulative percentage mortality of the parasite. The 9, 12 and 15h median effective concentration (EC_{50}) of azadirachtin against juvenile *Argulus* was 57.64, 37.78 and 20.24 mg L^{-1} respectively (Table 10 and Fig 4) which was slightly lower as compared to EC_{50} against adult *Argulus.* The *in vitro* antiparasitic

efficacy was calculated and represented in Table 11 showing that the antiparasitic efficacy of azadirachtin test solutions viz. T_1 (25 mg L^{-1}), T_2 (50 mg L^{-1}), T_3 (75 mg L^{-1}), T_4 (100 mg L^{-1}) and T_5 (125 mg L^{-1}) was 50%, 66.67%, 83.33%, 100% and 100% respectively in 12h and 50%, 83.33%, 100%, 100% and 100% respectively in 15h against juvenile *Argulus*. No mortality of any parasite was encountered from the control group in 36h.

4.4.1.3 *In vitro* ovicidal effect of azadirachtin on *Argulus* eggs

The hatching of *Argulus* eggs was affected at azadirachtin concentrations above 100 mg L^{-1}. The first twitching movement of nauplius inside the eggs was noticed on day 6 in control and 25 mg L^{-1} treatment groups. The first hatching of *Argulus* nauplii was observed on day 8 after the setup. The hatching continued upto day 14 when 100 % hatching was achieved in the control group and 25 and 50 mg L^{-1} treatment groups. Hatching of *Argulus* eggs ceased from fourteenth day onwards with no new hatching observed in any treatment groups upto the next seven days despite of maintaining the same conditions for hatching. Less than 50% eggs were hatched in T_6 (150 mg L^{-1}) and T_7 (175 mg L^{-1}) treatment groups in fourteen days. The eggs in T_5 (125 mg L^{-1}), T_6 (150 mg L^{-1}) and T_7 (175 mg L^{-1}) treatment groups had become very unclear by the fourteenth day with developed nauplius visible inside the eggs but were unable to hatch. The hatched nauplius was able to survive for 36-48 h in the control group, 12-24 h in T_1 (25 mg L^{-1}) and T_2 (50 mg L^{-1}) treatment groups but less than 6 h in rest of the treatment groups.

The cumulative average hatching and percentage hatching of *Argulus* eggs is represented in Table 12. The percentage hatching is also shown in Fig 5. *Argulus* eggs from control and T_7 (175 mg L^{-1}) treatment groups, before and after fourteen days are represented in Plate 7 along with a healthy nauplius of control group and a dead nauplius from treatment group. The eggs in the treatment group was unclear with a developed nauplius which was unable to hatch while all the eggs had hatched in the control group.

Table 6: (*In vitro* study) cumulative average mortality (Cav. M) and cumulative percentage mortality (C% M) of adult *Argulus* spp. treated with different concentrations of Aza (EC 21.5%)

Treatments	Time 6 h		Time 9 h		Time 12 h		Time 15 h		Time 18 h	
	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M
T0	0	0	0	0	0	0	0	0	0	0
T1	3	16.67	8	44.44	9	50	11	61.11	18	100
T2	6	33.33	10	55.56	12	66.67	15	83.33	18	100
T3	6	33.33	10	55.56	14	77.78	18	100	18	100
T4	6	33.33	12	66.66	18	100	18	100	18	100
T5	6	33.33	15	83.33	18	100	18	100	18	100

Table 7: Median Effective Concentration (EC50) of azadirachtin (EC 21.5%) against adult *Argulus* spp. at different time intervals

Time (Hours)	6	9	12	15	18
EC 50 (mg L^{-1})	146.93	61.29	39.04	27.18	Not determined

Table 8: *In vitro* antiparasitic efficacy (%) of azadirachtin (21.5%) against Adult *Argulus*

Treatments (mg L^{-1})	Time					
	3 h	6 h	9 h	12 h	15 h	18 h
T0	-	-	-	-	-	-
T1	0	16.67	44.44	50	61.11	100
T2	0.00	33.33	55.56	66.67	83.33	100
T3	0.00	33.33	55.56	77.78	100	100
T4	0.00	33.33	66.67	100	100	100
T5	16.67	33.33	83.33	100	100	100

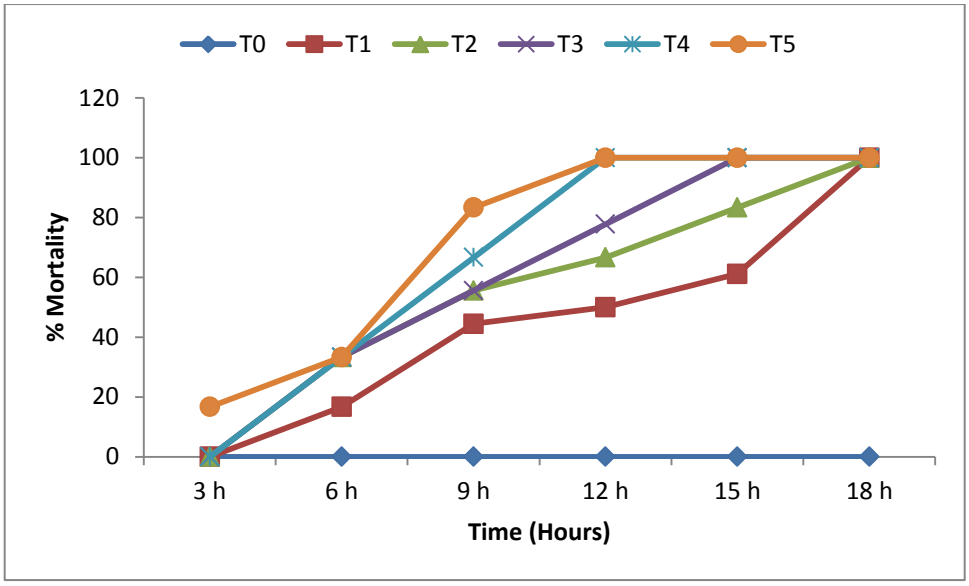


Fig 1: Cumulative percentage mortality of adult *Argulus* spp.at different concentrations of azadirachtin (21.5%) with respect to time.

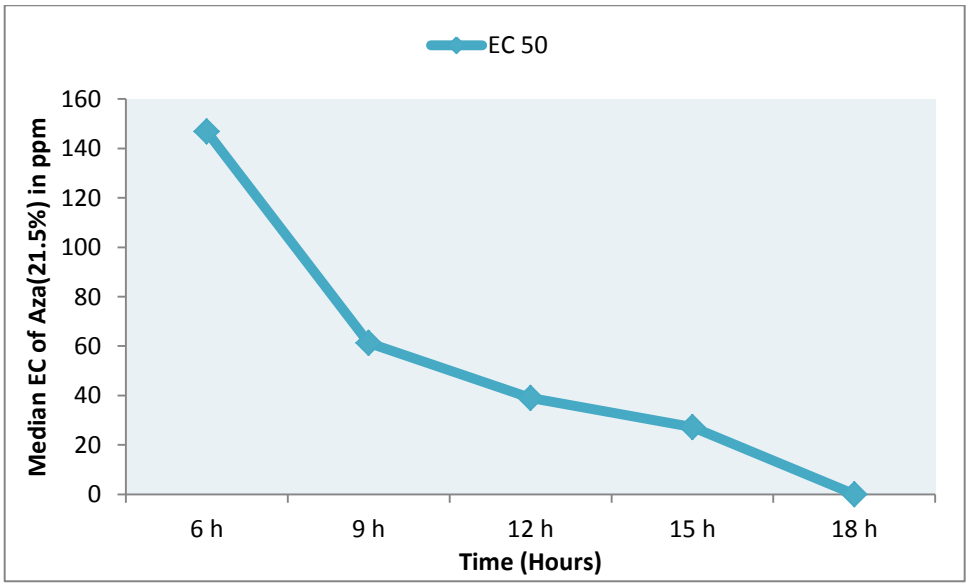


Fig 2: EC_{50} (mg L^{-1}) of azadirachtin (21.5%)against Adult *Argulus* spp.

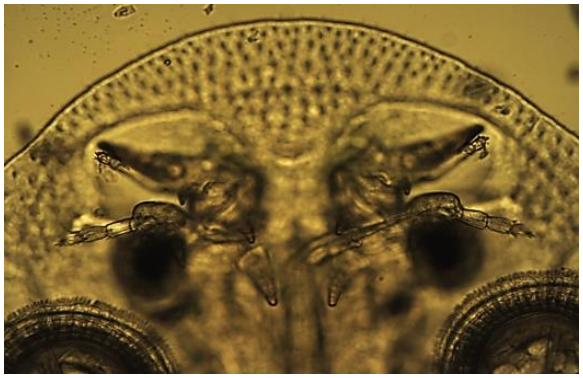
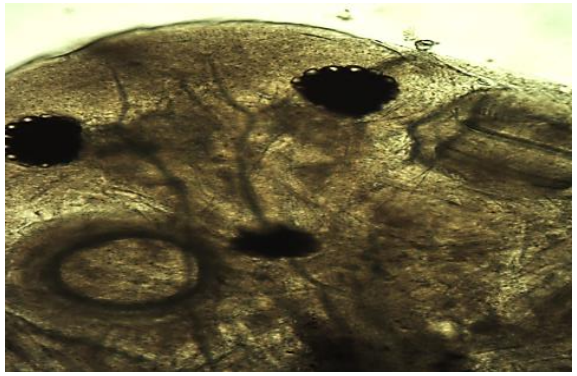
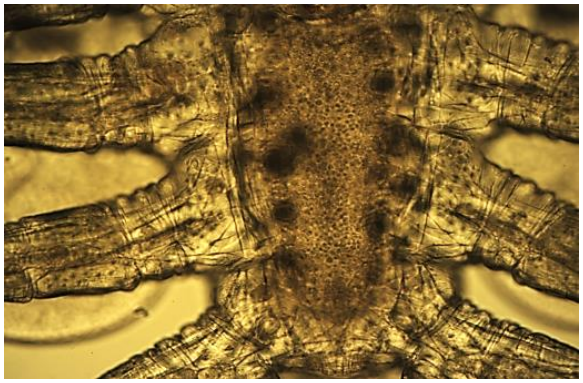
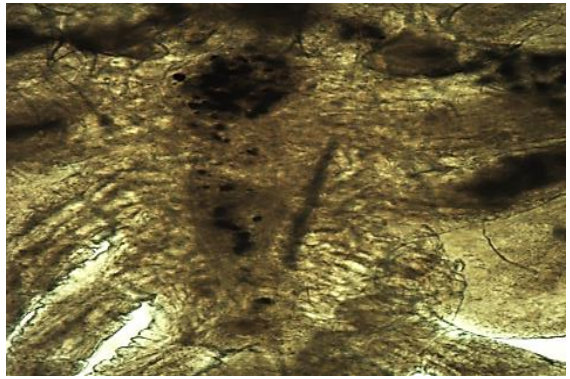
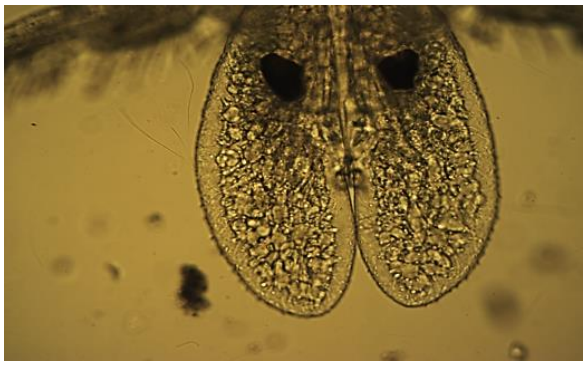







Plate 6. Adult *Argulus* before azadirachtin treatment (left) and after azadirachtin treatment (right)

Table 9: (*In vitro* study) cumulative average mortality (Cav. M) and cumulative percentage mortality (C% M) of juvenile *Argulus* spp. treated with different concentration of Aza (EC 21.5%)

Treatments	Time 3 h		Time 6 h		Time 9 h		Time 12 h		Time 15 h		Time 18 h	
	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M
T0	0	0	0	0	0	0	0	0	0	0	0	0
T1	0	0	2	11.11	4	22.22	9	50	15	83.33	18	100
T2	1	5.56	3	16.67	10	55.56	12	66.67	18	100	18	100
T3	1	5.56	4	22.22	13	72.22	15	83.33	18	100	18	100
T4	1	5.56	5	27.78	15	83.33	18	100	18	100	18	100
T5	2	11.11	5	27.78	16	88.89	18	100	18	100	18	100

Table 10: Median Effective Concentration (EC_{50}) of azadirachtin (EC 21.5%) against juvenile *Argulus* spp. at different time intervals

Time (Hours)	3	6	9	12	15	18
EC 50 (mg L^{-1})	242.67	167.69	57.64	37.78	20.24	Cannot be determined

Table 11: *In vitro* antiparasitic efficacy (%) of azadirachtin (EC 21.5%) against Juvenile *Argulus*

Treatments (mg L^{-1})	Time					
	3 h	6 h	9 h	12 h	15 h	18 h
T0	-	-	-	-	-	-
T1	0	11.11	22.22	50	50	100
T2	5.56	16.67	55.56	66.67	83.33	100
T3	5.56	22.22	72.22	83.33	100	100
T4	5.56	27.78	83.33	100	100	100
T5	11.11	27.78	88.89	100	100	100

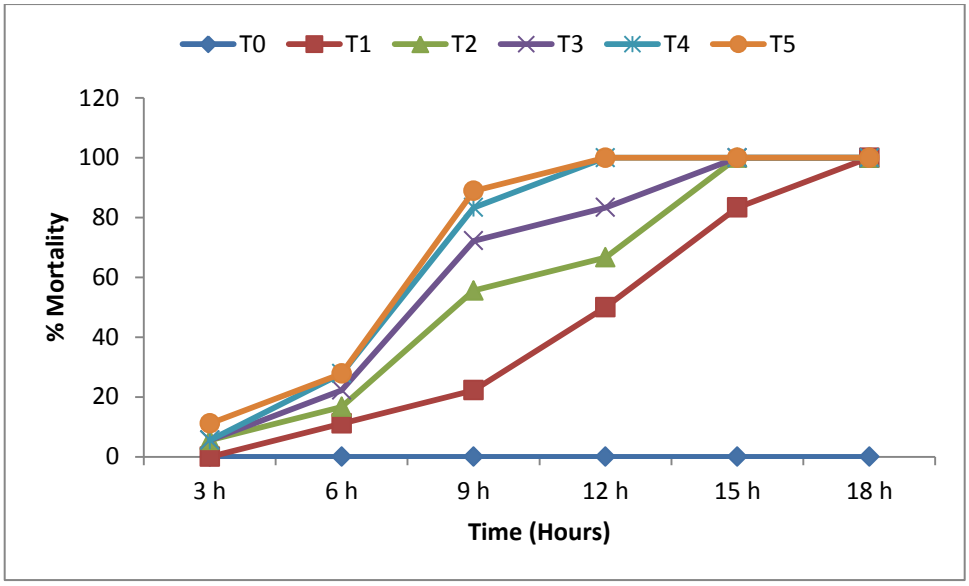


Fig 3. Cumulative percentage mortality of juvenile *Argulus* spp.at different concentrations of azadirachtin (21.5%) with respect to time

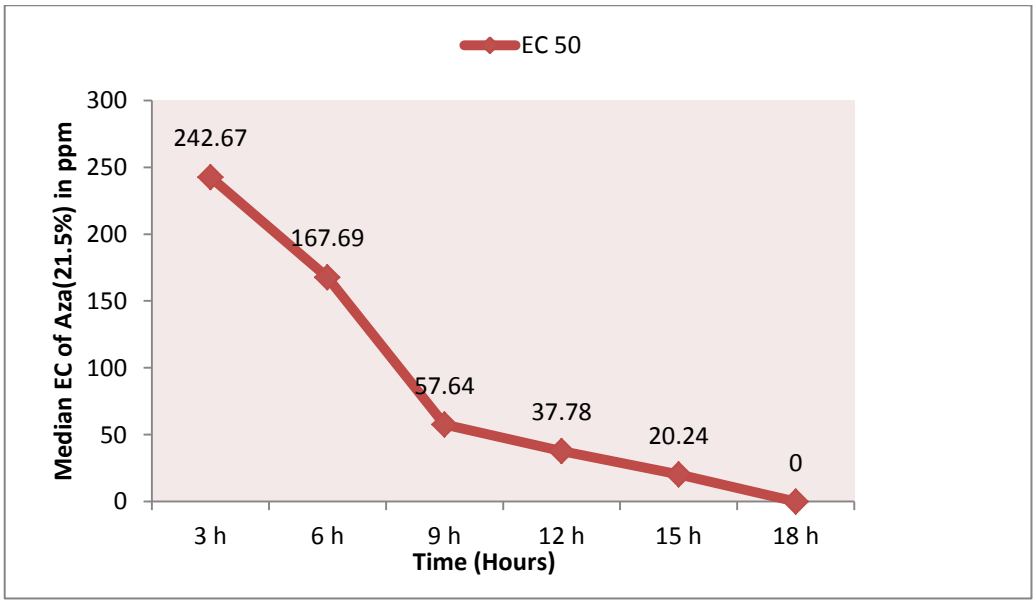


Fig 4. EC_{50} (mg L^{-1}) of azadirachtin (21.5%) against juvenile *Argulus* spp.

Table 12: Cumulative average hatching (Cav. hat) and cumulative percentage hatching (C% hat) of *Argulus* eggs exposed to different concentrations of Aza (EC 21.5%) with respect to the number of days

Treatments	Day 8		Day 9		Day 10		Day 11		Day 12		Day 13		Day 14	
	Cav. hat.	C% hat.	Cav. hat.	C% hat.	Cav. hat.	C% hat.	Cav. hat.	C% hat.	Cav. hat.	C% hat.	Cav. hat.	C% hat.	Cav. hat.	C% hat.
T0	7	23.33	11	36.67	18	60	24	80	24	80	30	100	30	100
T1	3	10	9	30	16	53.33	20	66.67	23	76.67	29	96.67	30	100
T2	3	10	8	26.67	12	40	16	53.33	18	60	27	90	30	100
T3	2	6.67	6	20	9	30	15	50	15	50	23	76.67	28	93.33
T4	2	6.67	5	16.67	8	26.67	13	43.33	14	46.67	20	66.67	22	73.33
T5	2	6.67	5	16.67	6	20	8	26.67	12	40	17	56.67	17	56.67
T6	0	0	2	6.67	4	13.33	5	16.67	8	26.67	13	43.33	13	43.33
T7	0	0	1	3.33	2	6.67	2	6.67	5	16.67	9	30	12	40

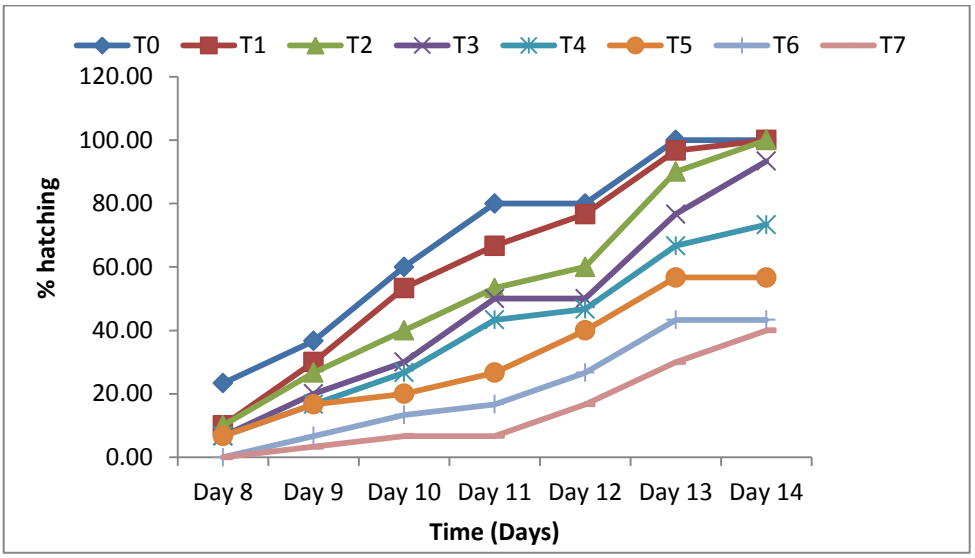
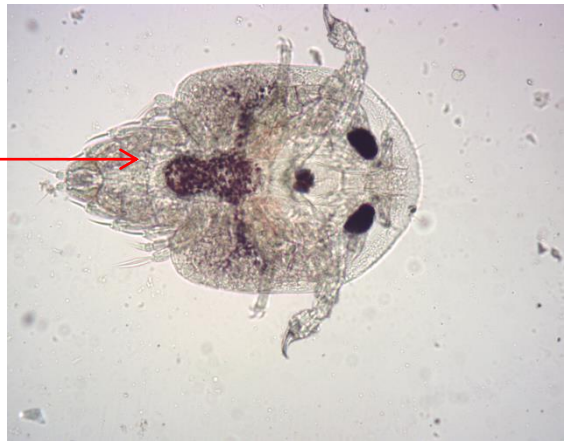
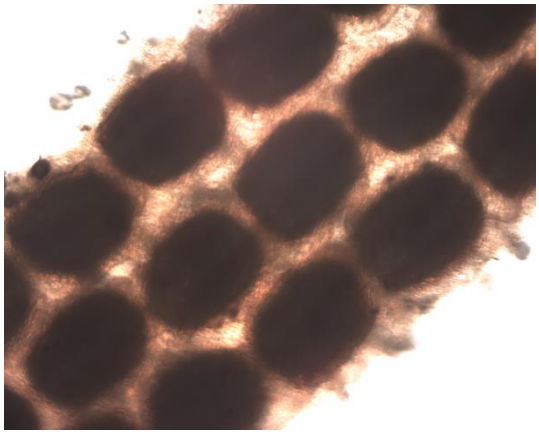


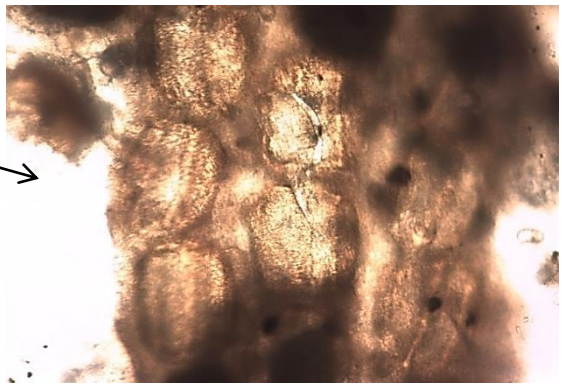
Fig 5. Cumulative percentage hatching of *Argulus* eggs at different time intervals exposed to different concentrations of azadirachtin (EC 21.5%) solution.

one day old *Argulus* nauplius.



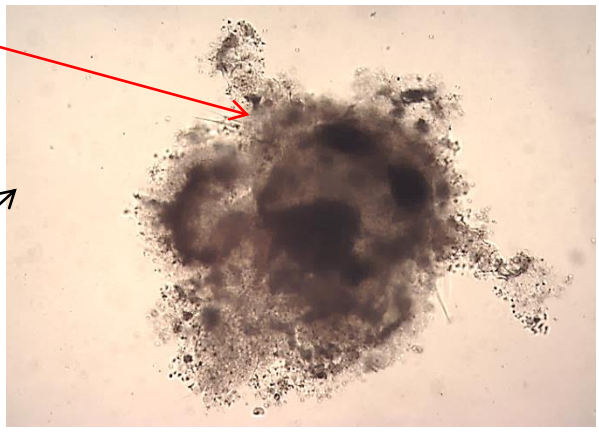


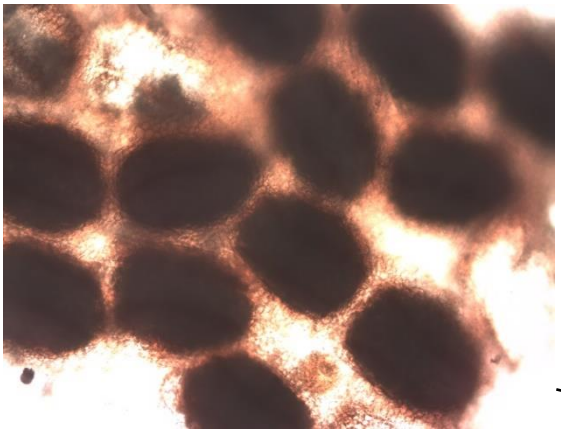
After 14 days



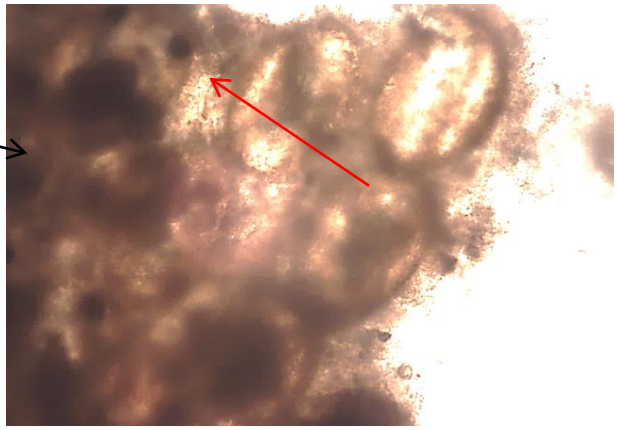
Control eggs Day 1

Argulus nauplius dead in 175 mg L^{-1} aza solution





After 14 days



Eggs in 175 mg L^{-1} aza solution (Day 1)

Plate 7. Comparison of *Argulus* eggs in control and Aza solution on day 1 and day 14.

4.4.1.4 *In vitro* antiparasitic effect of azadirachtin against theronts of *I. multifiliis*

The present *in vitro* study of azadirachtin against theronts showed that there was an increase in mortality with an increase in dose of azadirachtin. The cumulative average and percentage mortality of theronts was observed and recorded at different time intervals (Table 13 and Fig. 6). The median effective concentration (EC_{50}) of azadirachtin against theronts for 6, 9 and 12h was obtained as 31.424, 20.944 and 11.838mg L^{-1} respectively (Table 14 and Fig. 7). The *in vitro* antiparastic efficacy of azadirachtin against theronts, given in Table 15 was 11.67%, 31.33%, 35%, 58.33% and 100% respectively in 6h and 19.33%, 67.67%, 70%, 86% and 100% respectively in 9h at concentrations of T_1 (9.52 mg L^{-1}), T_2 (19.05 mg L^{-1}), T_3 (28.57 mg L^{-1}), T_4 (38.09 mg L^{-1}) and T_5 (47.61 mg L^{-1}) showing that it is potent against theronts.

No mortality of theronts was observed upto 36h and morphology of the theronts before and after treatment was noted (Plate 8).

4.4.1.5 *In vitro* antiparasitic effect of azadirachtin against *Dactylogyrus* sp

A dose dependent mortality of *Dactylogyrus* was observed when treated with different concentrations of azadirachtin in the present *in vitro* study. The cumulative average and percentage mortality of the parasite was observed and recorded at different time intervals (Table 16 and Fig 8). The median effective concentration (EC_{50}) of azadirachtin against *Dactylogyrus* is represented in Table 17 and Fig 9 with the 2 , 3 and 4 EC_{50} being 21.651, 14.705 and 6.088 mg L^{-1} respectively. Similarly, the *in vitro* antiparasitic efficacy of azadirachtin against *Dactylogyrus* at different concentrations viz. T_1 (1 mg L^{-1}), T_2 (5 mg L^{-1}), T_3 (10 mg L^{-1}), T_4 (15 mg L^{-1}), T_5 (20 mg L^{-1}), T_6 (25 mg L^{-1}) and T_7 (30 mg L^{-1}) was 10%, 20%, 30%, 40%, 76.7%, 83.33% and 100% respectively in 3h and 42.85%, 53.57%, 67.85%, 75%, 78.57%, 100% and 100% respectively in 4h (Table 18).

Significant mortality of *Dactylogyrus* in control started after 8h. The morphology of parasite, before and after treatment was noted (Plate 9).

Table 13: (*In vitro* study) cumulative average mortalit (Cav. M) and cumulative percentage mortality (C% M) of theronts treated with different concentration of Aza (EC 21.5%)

Treatments	Time 3 h		Time 6 h		Time 9 h		Time 12 h		Time 15 h		Time 18 h	
	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M
T0	0	0	0	0	0	0	0	0	0	0	0	0
T1	16	5.33	35	11.67	58	19.33	64	21.33	110	36.67	180	60
T2	52	17.33	94	31.33	203	67.67	300	100	300	100	300	100
T3	65	21.67	105	35	210	70	300	100	300	100	300	100
T4	86	28.67	175	58.33	258	86	300	100	300	100	300	100
T5	182	60.67	300	100	300	100	300	100	300	100	300	100

Table 14: Median Effective Concentration (EC_{50}) of azadirachtin (EC 21.5%) against theronts at different time intervals

Time (Hours)	3	6	9	12	15	18
EC 50 (mg L^{-1})	46.802	31.424	20.944	11.838	10.82	9.42

Table 15: *In vitro* antiparasitic efficacy (%) of azadirachtin (21.5%) against theronts

Treatments (mg L^{-1})	Time					
	3 h	6 h	9 h	12 h	15 h	18 h
T0	-	-	-	-	-	-
T1	5.33	11.67	19.33	21.33	36.67	60
T2	17.33	31.33	67.67	100	100	100
T3	21.67	35	70	100	100	100
T4	28.67	58.33	86	100	100	100
T5	60.67	100	100	100	100	100

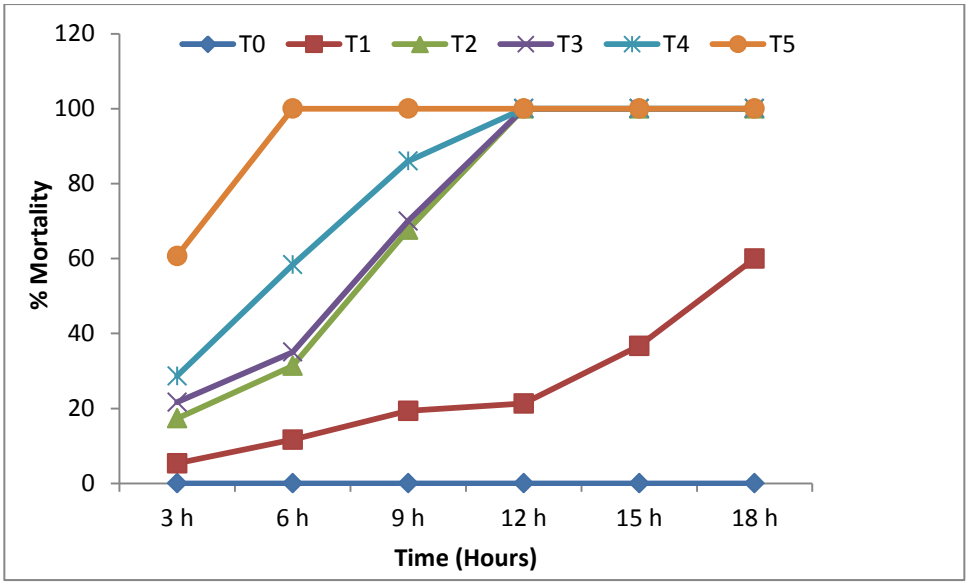


Fig 6. Cumulative percentage mortality of theronts at different concentrations of azadirachtin (EC 21.5%) with respect to time.

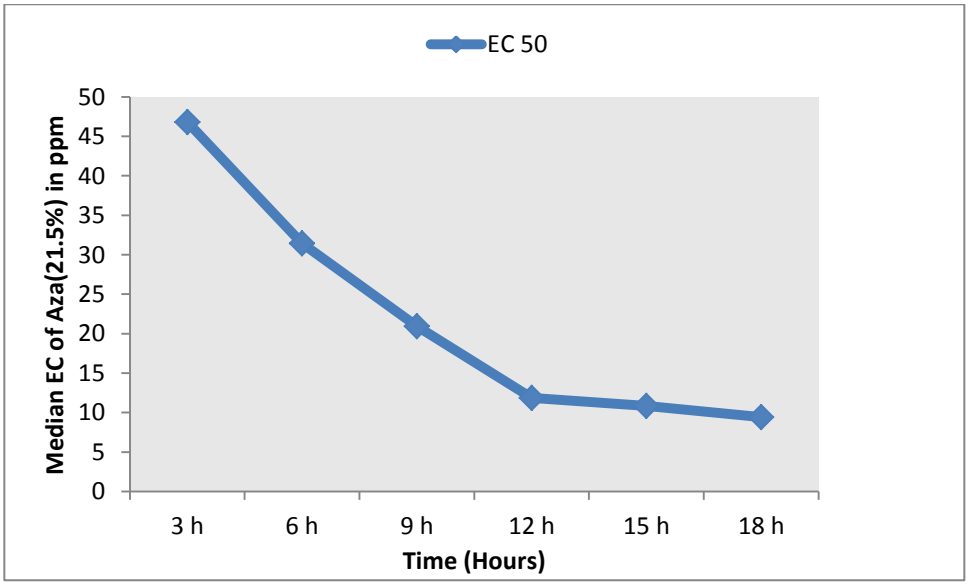


Fig 7. EC_{50} (mg L^{-1}) of azadirachtin (21.5%) against theronts.

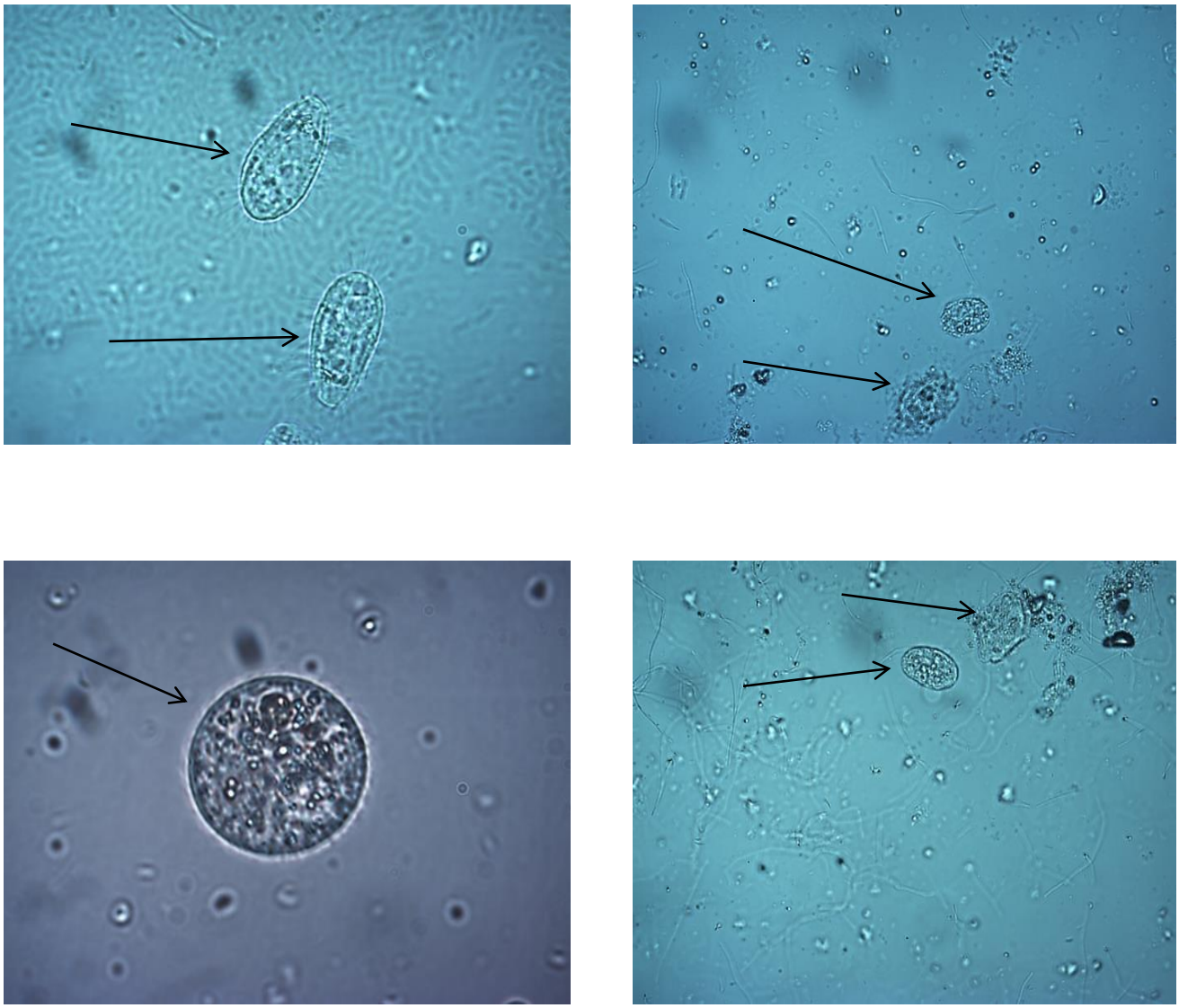


Plate 8. Theronts before azadirachtin treatment (left) and after azadirachtin treatment (right).

Table 16: (*In vitro* study) cumulative average mortality (Cav. M) and cumulative percentage mortality (C% M) of *Dactylogyrus* sp. treated with different concentration of Aza (EC 21.5%)

Treatments	Time 1 h		Time 2 h		Time 3 h		Time 4 h		Time 5 h	
	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M
T0	0	0	0	0	0	0	2	6.67	2	6.67
T1	0	0	0	0	3	10	14	46.67	26	86.67
T2	0	0	5	16.67	6	20	17	56.67	30	100
T3	0	0	4	13.33	9	30	21	70	30	100
T4	2	6.67	7	23.33	12	40	23	76.67	30	100
T5	7	23.33	12	40	23	76.67	24	80	30	100
T6	9	30	15	50	25	83.33	30	100	30	100
T7	13	43.33	27	90	30	100	30	100	30	100

Table 17: Median Effective Concentration (EC_{50}) of azadirachtin (EC 21.5%) against *Dactylogyrus* sp. at different time intervals

Time (Hours)	1	2	3	4	5
EC 50 (mg L^{-1})	30.272	21.651	14.705	6.088	Not determined

Table 18: *In vitro* antiparasitic efficacy (%) of azadirachtin (EC 21.5%) against *Dactylogyrus*

Treatments (mg L^{-1})	Time				
	1 h	2 h	3 h	4 h	5 h
T0	-	-	-	-	-
T1	0	0	10	42.85	85.71
T2	0	16.67	20	53.57	100
T3	0	13.33	30	67.85	100
T4	6.67	23.33	40	75	100
T5	23.33	40	76.67	78.57	100
T6	30	50	83.33	100	100
T7	43.33	90	100	100	100

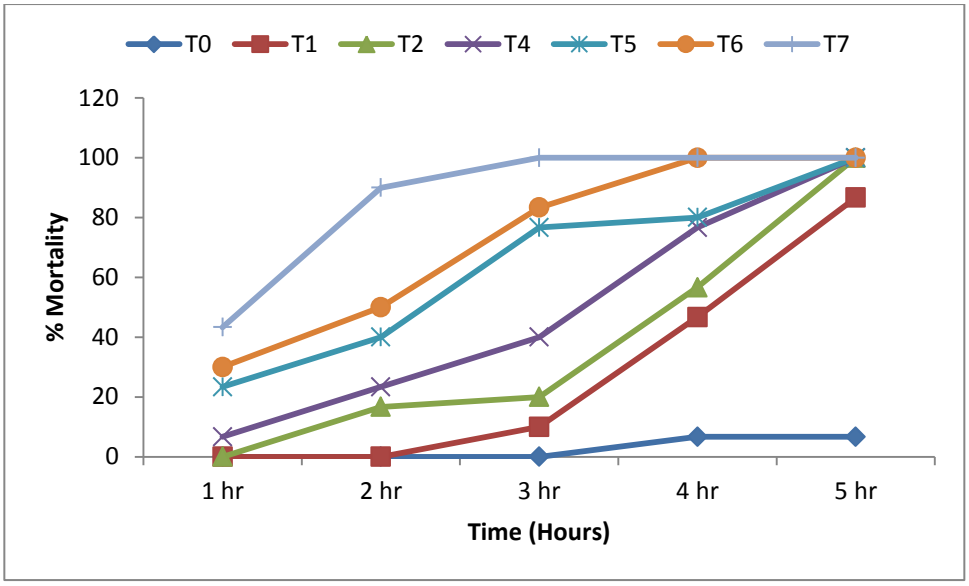


Fig 8. Cumulative percentage mortality of *Dactylogyrus* sp.at different concentrations of azadirachtin (EC 21.5%) with respect to time.

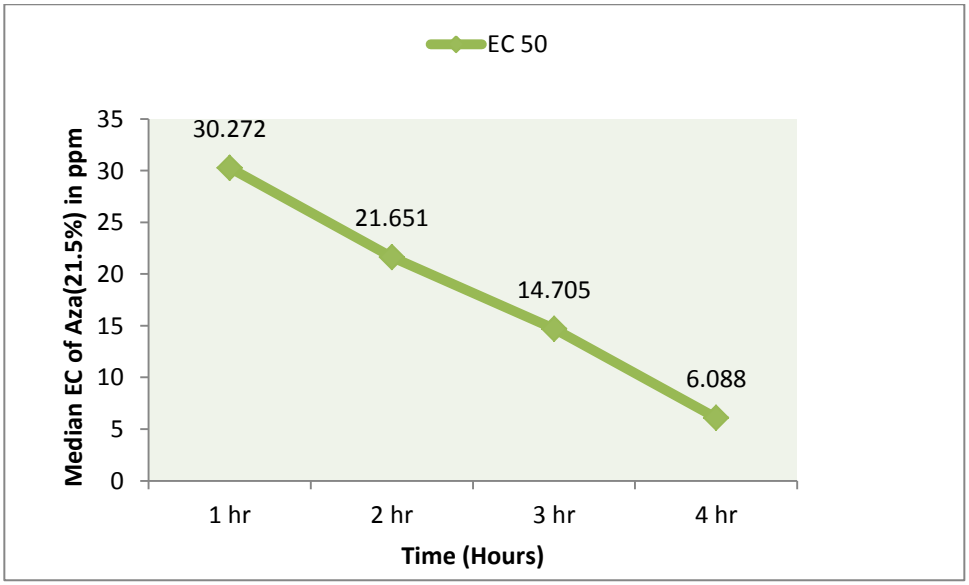
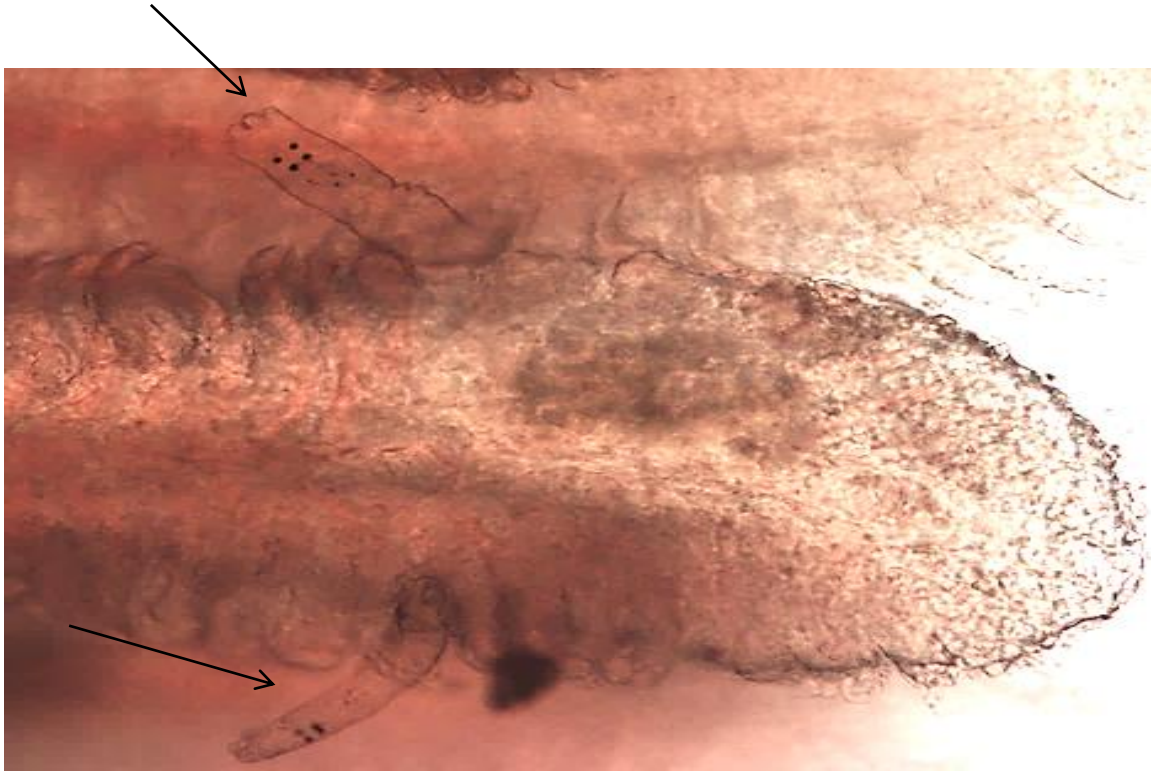
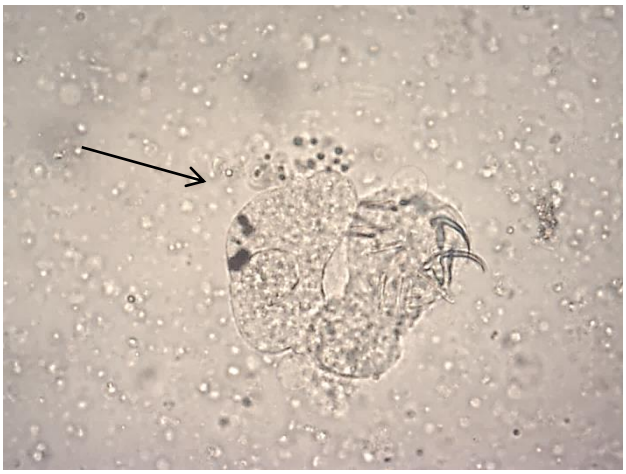
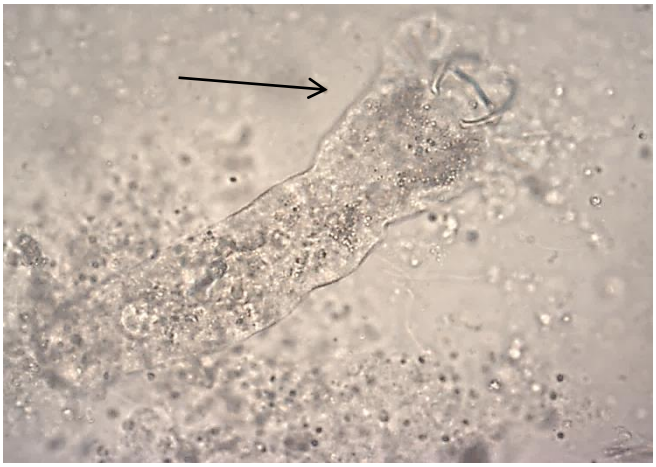


Fig 9. EC_{50} (mg L^{-1}) of azadirachtin (EC 21.5%) against *Dactylogyrus* sp.



***Dactylogyrus* before treatment with azadirachtin**





***Dactylogyrus* after treatment with azadirachtin**

Plate 9. *Dactylogyrus* sp. before and after azadirachtin treatment

4.4.1.6 Acute toxicity of azadirachtin for goldfish

A dose dependent mortality of goldfish was observed when exposed to different concentrations of azadirachtin solution. Due to the mortality of fish at lower concentrations of azadirachtin, the median lethal concentration (LC_{50}) of goldfish was recorded by exposing fish to two different ranges of azadirachtin concentrations. The cumulative percentage mortality of fish upto 15h and 96h are represented in Fig 10. and Fig 11 respectively showing 100% mortality of goldfish in 12h at 50 mg L^{-1} concentration of azadirachtin and 48h at 10 mg L^{-1} concentration of azadirachtin respectively. The median lethal concentration (LC_{50}) of azadirachtin against goldfish was observed and recorded for 15 h and 96 h respectively (Table 19 and Table 20). The LC50 of azadirachtin against goldfish for 9, 12 and 15h was 39.58, 25.07 and 20.48 mg L^{-1} respectively while the LC50 for 48, 72 and 96h was 6.1, 5.05 and 4.68 mg L^{-1} respectively (Fig 12 and Fig 13)

No mortality of goldfish was recorded in the control group of fish during the acute toxicity test period upto 96 h.

4.4.1.7 Therapeautic index of azadirachtin

The therapeautic index of azadirachtin was calculated and represented in Fig 14. The three hours estimated therapeautic value of azadirachtin was found to be 5.33 for *Dactylogyrus* sp. and 1.67 for theronts while the nine hours estimated therapeautic value of azadirachtin against adult and juvenile *Argulus* was 0.65 and 0.69 respectively.

Fig 15. shows the median effective concentration (EC_{50}) of azadirachtin against common ectoparasites of goldfish along with the median lethal concentration (LC_{50}) for goldfish.

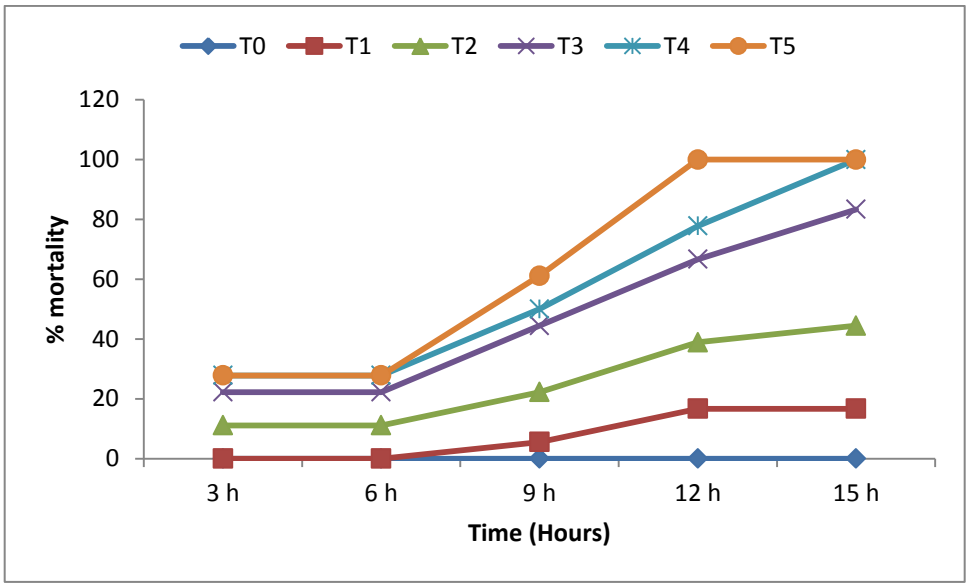


Fig 10. Cumulative percentage mortality of goldfish with respect to time at three hours interval upto 15 hour in azadirachtin solution

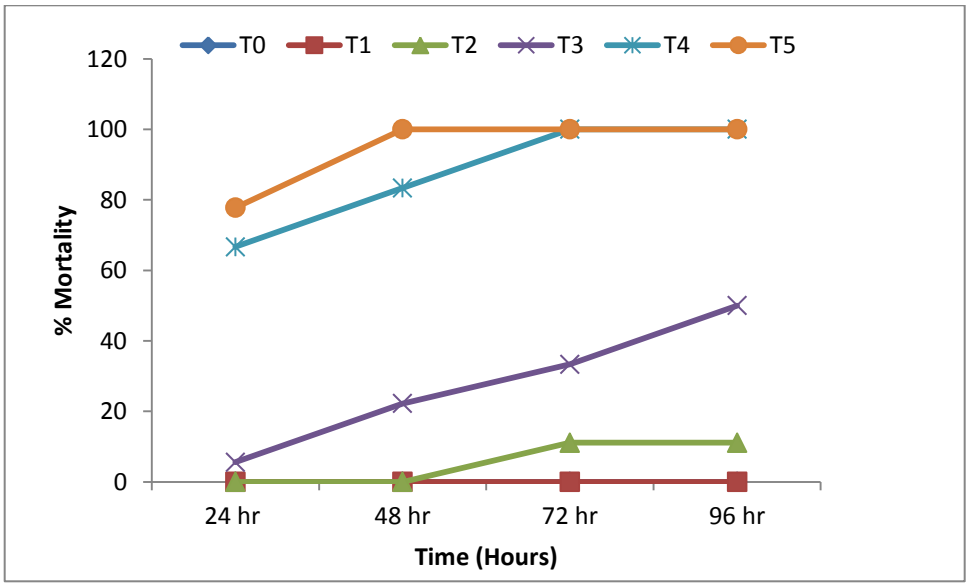


Fig 11. Cumulative percentage mortality of goldfish with respect to time at Twenty four hours interval upto 96 hours in azadirachtin solution

Table 19: Median lethal concentration (LC_{50}) of azadirachtin (EC 21.5%) against goldfish measured at three hours interval upto 15 hours

Time (Hours)	3	6	9	12	15
LC 50 (mg L^{-1})	78.36	59.47	39.58	25.07	20.48

Table 20: Median lethal concentration (LC_{50}) of azadirachtin (EC 21.5%) against goldfish measured at twenty four hours interval upto 96 hours

Time (Hours)	24	48	72	96
LC 50 (mg L^{-1})	7.62	6.1	5.05	4.68

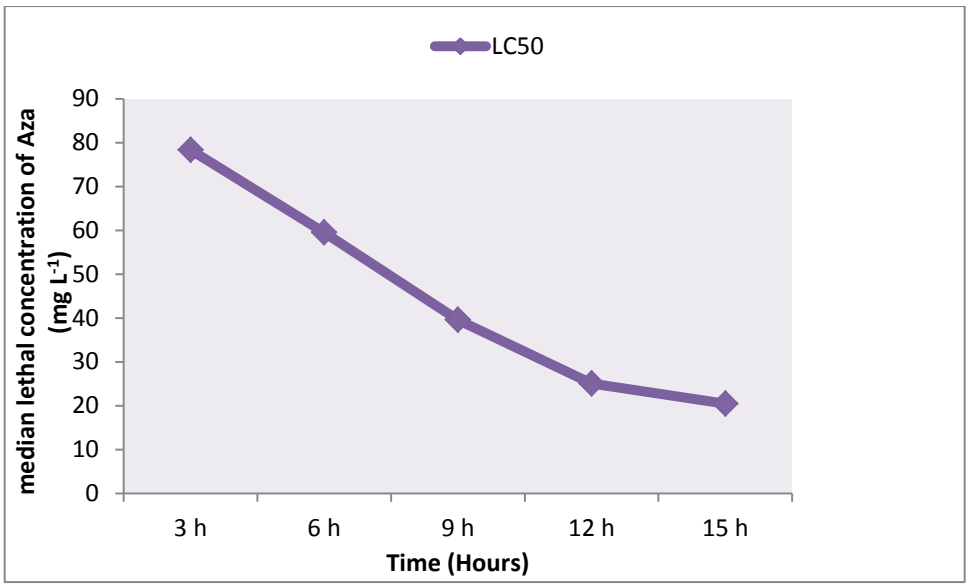


Fig 12. Median lethal concentration (LC_{50}) of azadirachtin (EC 21.5%) for goldfish upto 15 hours

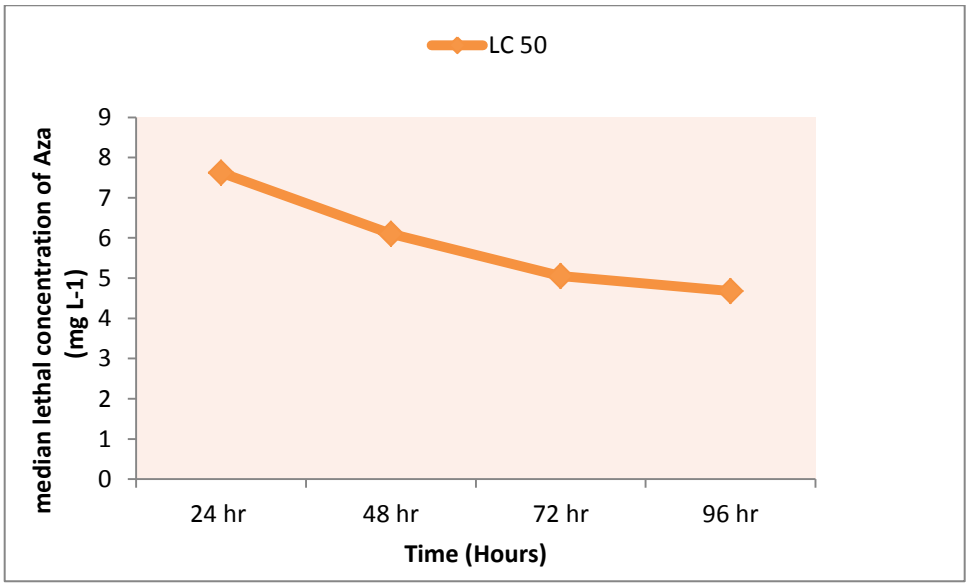


Fig 13. Median lethal concentration (LC_{50}) of azadirachtin (EC 21.5%) for goldfish upto 96 hours

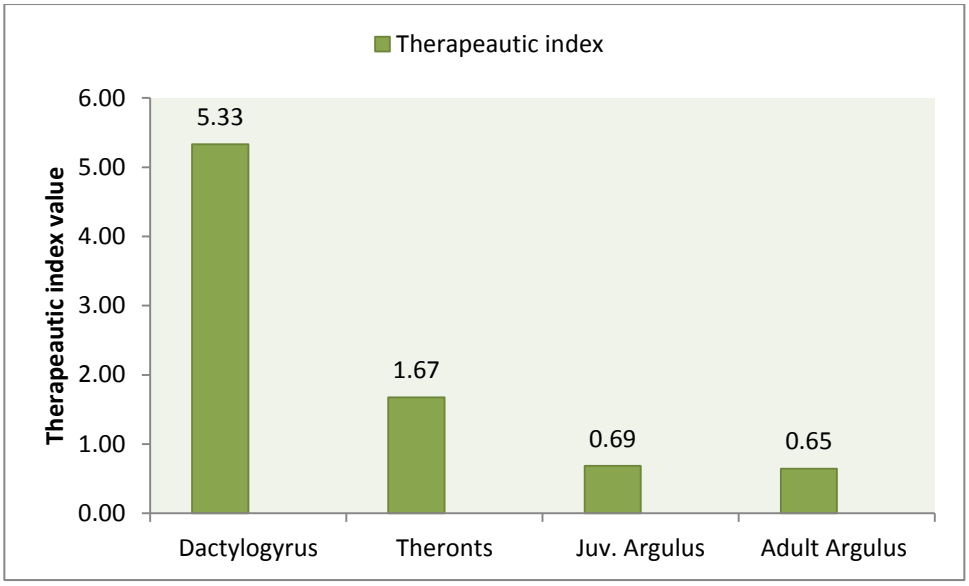


Fig 14. Therapeautic index value for azadirachtin (EC 21.5%) against common goldfish ectoparasites

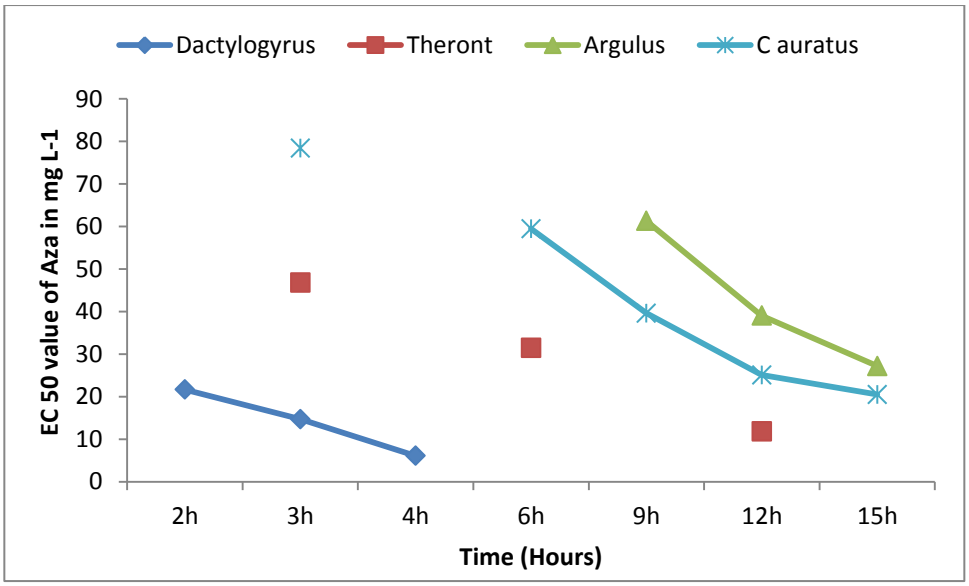


Fig 15. Comparative graph showing the median effective concentration (EC_{50}) values of azadirachtin against common goldfish ectoparasites and median lethal concentration (LC_{50}) values for goldfish to understand the use of azadirachtin for *in vivo* treatment of ectoparasites in goldfish.

4.4.2 *In vitro* antiparasitic effect of allicin (EC 100%) against common ectoparasites of goldfish

4.4.2.1 *In vitro* antiparasitic effect of allicin against *Argulus* spp.

The present *in vitro* study showed an increase in mortality of *Argulus* with corresponding increase in the dose of allicin. The average and percentage mortality of *Argulus* was observed and recorded in Table 21. The percentage mortality is also represented in Fig 16. The median effective concentration (EC_{50}) of allicin against *Argulus* is represented in Table 22 and Fig 17 with 4.89, 3.19 and 1.87 mg ml^{-1} being the EC_{50} value for 9, 12 and 15h respectively. The *in vitro* antiparasitic efficacy of allicin against *Argulus* at different concentrations viz. T_1 (2 mg ml^{-1}), T_2 (2.5 mg ml^{-1}), T_3 (3 mg ml^{-1}), T_4 (3.5 mg ml^{-1}) and T_5 (4 mg ml^{-1}) was 77.78%, 77.78%, 88.89%, 94.44% and 100% respectively in 18h and 88.89%, 88.89%, 94.44%, 100% and 100% respectively in 21h (Table 23).

No mortality of parasites was encountered in the control group upto 36h.

4.4.2.2 *In vitro* antiparasitic effect of allicin against *Dactylogyrus* sp.

The present *in vitro* study showed a dose dependent mortality of *Dactylogyrus* exposed to different solutions of allicin. The percentage mortality of the parasite is represented in Fig 18 while the average and percentage mortality is represented in Table 24. The median effective concentration (EC_{50}) against *Dactylogyrus* was observed and shown in Table 25. The 2, 3 and 4h EC_{50} of allicin against *Dactylogyrus* was 1.8, 1.28 and 0.85 mg ml^{-1} respectively (Fig 19). Similarly antiparasitic efficacy of allicin was calculated against *Dactylogyrus* (Table 26). The *in vitro* antiparasitic efficacy of allicin at different concentrations viz. T_1 (1 mg ml^{-1}), T_2 (1.5 mg ml^{-1}), T_3 (2 mg ml^{-1}), T_4 (2.5 mg ml^{-1}), T_5 (3 mg ml^{-1}), T_6 (3.5 mg ml^{-1}) and T_7 (4 mg ml^{-1}) was 26.67%, 43.33%, 50%, 73.33%, 86.67% and 100% respectively in 2 h and 40%, 63.33%, 80%, 100%, 100%, 100% and 100% respectively in 3 h. Significant mortality of *Dactylogyrus* was observed in control after 8 h and the morphology of the parasite before and after treatment was noted (Plate 10).

Table 21: (*In vitro* study) cumulative average mortality (Cav. M) and cumulative percentage mortality (C% M) of *Argulus* spp. treated with different concentration of allicin (EC 100%)

Treatments	Time 6 h		Time 9 h		Time 12 h		Time 15 h		Time 18 h		Time 21 h		Time 24 h	
	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M
T0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1	0	0	1	5.56	5	27.78	12	66.67	14	77.78	16	88.89	18	100
T2	1	5.56	3	16.67	7	38.89	13	72.22	14	77.78	16	88.89	18	100
T3	2	11.11	3	16.67	9	50	15	83.33	16	88.89	17	94.44	18	100
T4	3	16.67	5	27.78	10	55.56	17	94.44	17	94.44	18	100	18	100
T5	3	16.67	5	27.78	11	61.11	17	94.44	18	100	18	100	18	100

Table 22: Median Effective Concentration (EC_{50}) of allicin (EC 100%) against *Argulus* spp. at different time intervals

Time (Hours)	6	9	12	15	18	21	24
EC 50 (mg ml^{-1})	5.33	4.89	3.19	1.87	1.69	1.46	1.04

Table 23: *In vitro* antiparasitic efficacy (%) of allicin (EC 100%) against *Argulus*

Treatments (mg ml^{-1})	Time						
	6 h	9 h	12 h	15 h	18 h	21 h	24 h
T0	-	-	-	-	-	-	-
T1	0	5.56	27.78	66.67	77.78	88.89	100
T2	5.56	16.67	38.89	72.22	77.78	88.89	100
T3	11.11	16.67	50	83.33	88.89	94.44	100
T4	16.67	27.78	55.56	94.44	94.44	100	100
T5	16.67	27.78	61.11	94.44	100	100	100

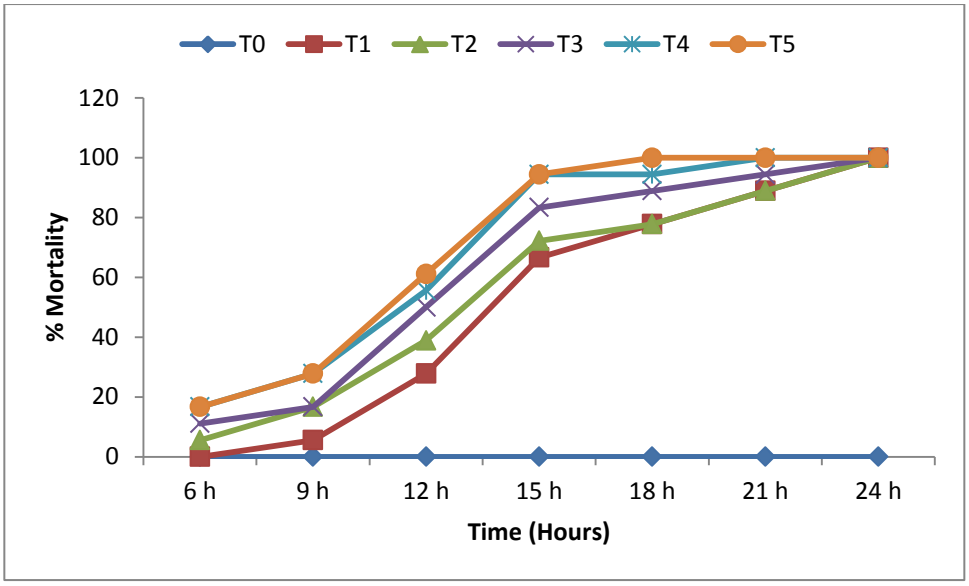


Fig 16. Cumulative percentage mortality of *Argulus* spp. at different concentrations of allicin (EC 100%) with respect to time.

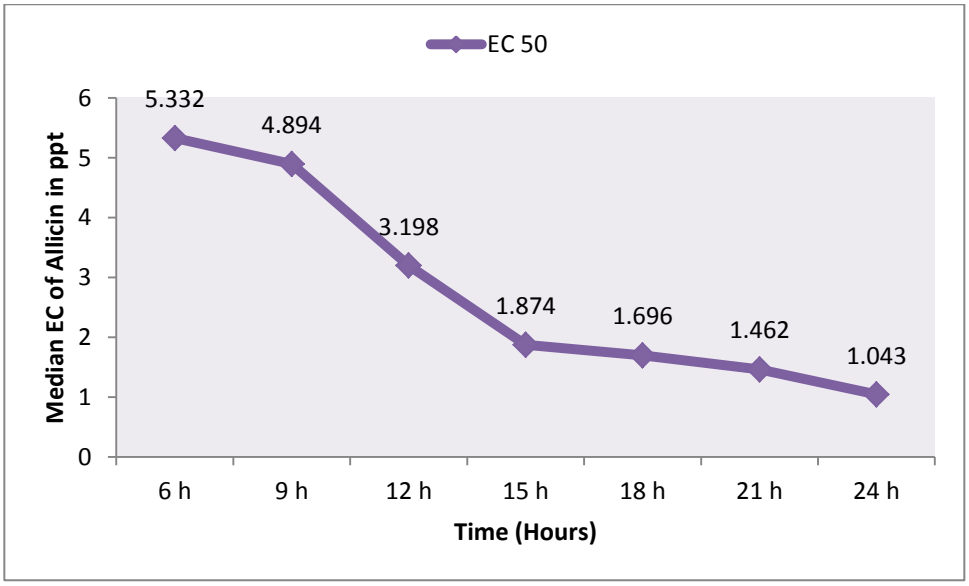


Fig 17. EC_{50} (mg ml^{-1}) of allicin (EC 100%) against *Argulus* spp.

Table 24: (*In vitro* study) cumulative average mortality (Cav. M) and cumulative percentage mortality (C% M) of *Dactylogyrus* sp. treated with different concentration of allicin (EC 100%)

Treatments	Time 1 h		Time 2 h		Time 3 h		Time 4 h		Time 5 h		Time 6 h	
	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M	Cav. M	C% M
T0	0	0	0	0	0	0	0	0	4	13.33	4	13.33
T1	0	0	8	26.67	12	40	21	70	25	83.33333	28	93.33
T2	3	10	13	43.33	19	63.33	28	93.33	30	100	30	100
T3	4	13.33	15	50	24	80	30	100	30	100	30	100
T4	7	23.33	22	73.33	30	100	30	100	30	100	30	100
T5	8	26.67	26	86.67	30	100	30	100	30	100	30	100
T6	11	36.67	30	100	30	100	30	100	30	100	30	100
T7	16	53.33	30	100	30	100	30	100	30	100	30	100

Table 25: Median Effective Concentration (EC_{50}) of allicin (EC 100%) against *Dactylogyrus* sp. at different time intervals

Time (Hours)	1	2	3	4	5	6
EC 50 (mg ml^{-1})	3.88	1.8	1.28	0.85	0.51	0.42

Table 26: *In vitro* antiparasitic efficacy (%) of allicin (EC 100%) against *Dactylogyrus*

Treatments (mg ml^{-1})	Time					
	1 h	2 h	3 h	4 h	5 h	6 h
T0	-	-	-	-	-	-
T1	0	26.67	40	70	80.77	0
T2	10	43.33	63.33	93.33	100	92.31
T3	13.33	50	80	100	100	100
T4	23.33	73.33	100	100	100	100
T5	26.67	86.67	100	100	100	100
T6	36.67	100	100	100	100	100
T7	53.33	100	100	100	100	100

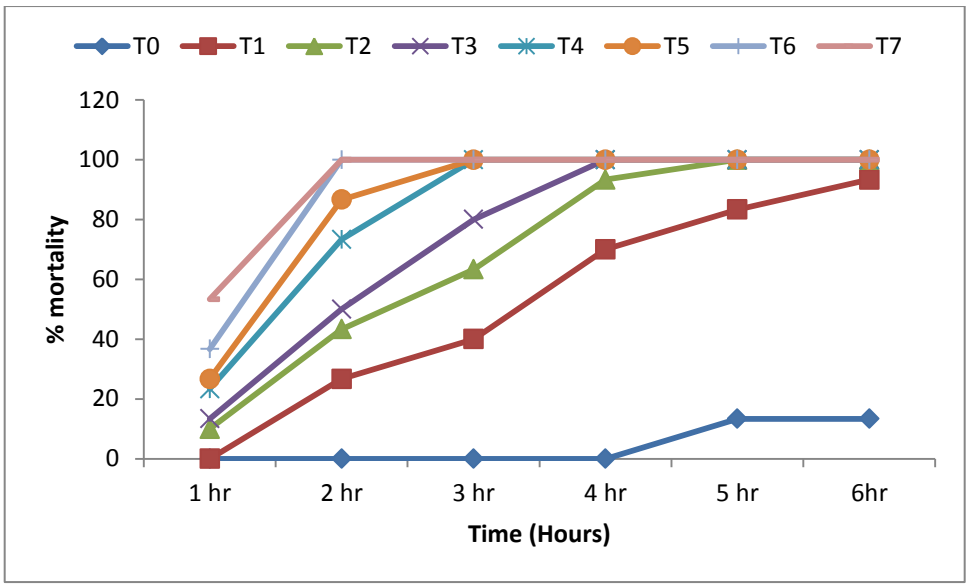


Fig 18. Cumulative percentage mortality of *Dactylogyrus* sp.at different concentrations of allicin (EC 100%) with respect to time

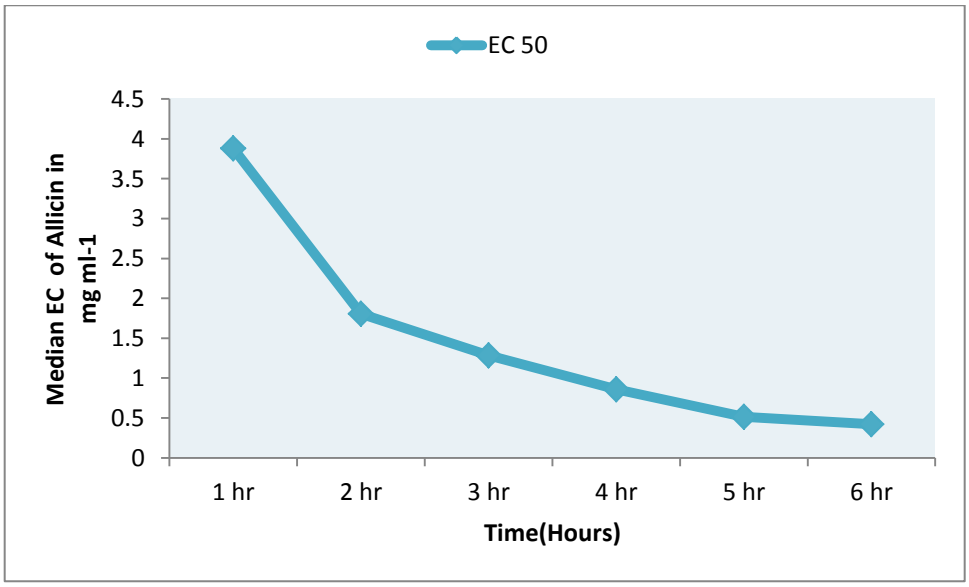
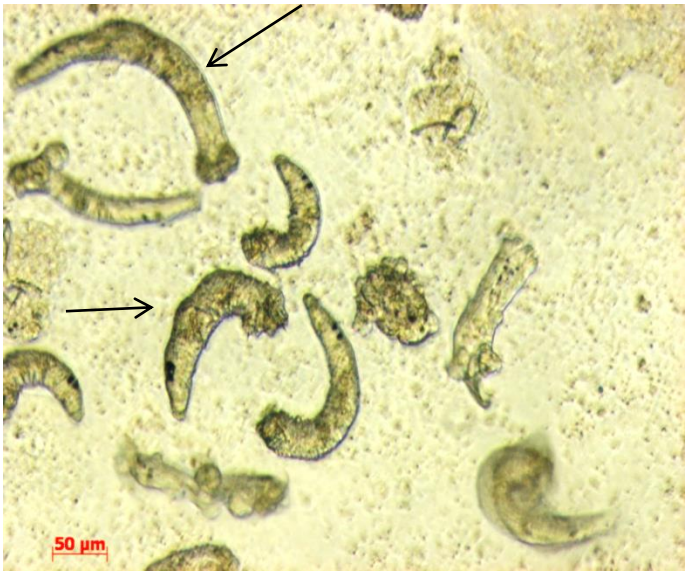
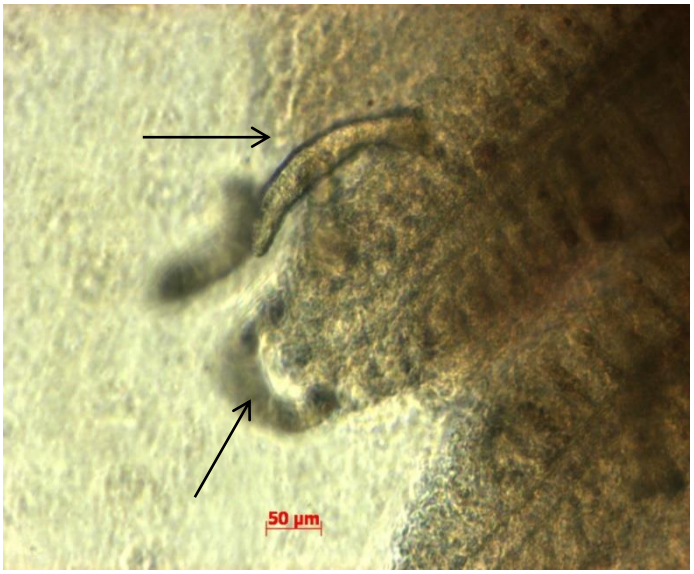
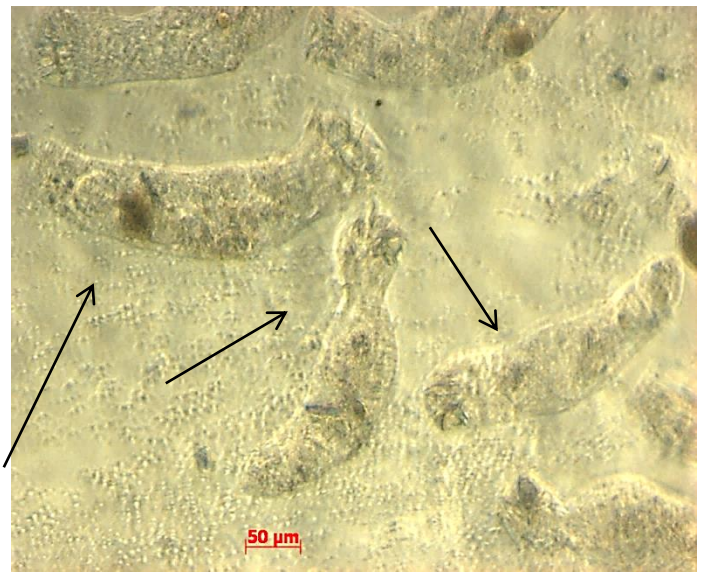


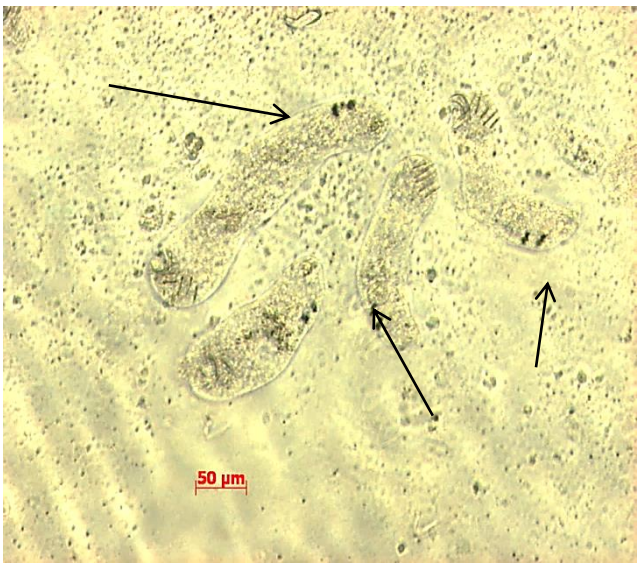
Fig 19. EC_{50} (mg L^{-1}) of allicin (EC 100%) against *Dactylogyrus* sp.





***Dactylogyrus* before treatment with allicin**





***Dactylogyrus* after treatment with allicin**

Plate 10. *Dactylogyrus* sp. before and after treatment with allicin

4.4.2.3 Acute tocxicity of allicin for goldfish

The median lethal concentration (LC_{50}) of allicin against goldfish was recorded and presented in Table 27 and Table 28 upto 15 h and 96 h respectively. The LC_{50} value for 9, 12 and 15h was 1.93, 1.72 and 1.63 mg ml^{-1} respectively and for 24 , 48 , 72 and 96h was 1.42, 1.17, 1.04, 0.81 mg ml^{-1} respectively (Fig 20 and 21) that showed a dose dependent mortality of goldfish. The percentage mortality was represented in Fig 22 and 23 for 15 h and 96 h respectively. The 100 % mortality of goldfish was observed in 24 h at allicin concentrations of 1.8 and 1.2 mg ml^{-1}.

No mortality of goldfish was observed in the control group during the 96 h test period.

4.4.2.4 Therapeautic index of allicin against common goldfish ectoparasites

The estimated therapeautic index of allicin against *Argulus* and *Dactylogyrus* is shown in Fig 24. The calculated six hours therapeautic value of allicin against *Dactylogyrus* was 5.57 which was higher than the therapeautic value of allicin against *Argulus* viz. 1.36 calculated for twenty four hours.

A comparative graph of median effective concentration (EC_{50}) and median lethal concentration (LC_{50}) of allicin against goldfish ectoparasites and goldfish respectively is represented in Fig 25.

Table 27: Median lethal concentration (LC_{50}) of allicin (EC 100%) against goldfish measured at three hours interval upto 15 hours

Time (Hours)	6	9	12	15
LC 50 (mg ml^{-1})	2.34	1.93	1.72	1.63

Table 28: Median lethal concentration (LC_{50}) of allicin (EC 100%) against goldfish measured at twenty four hours interval upto 96 hours

Time (Hours)	12	24	48	72	96
LC 50 (mg L^{-1})	1.71	1.42	1.17	1.04	0.81

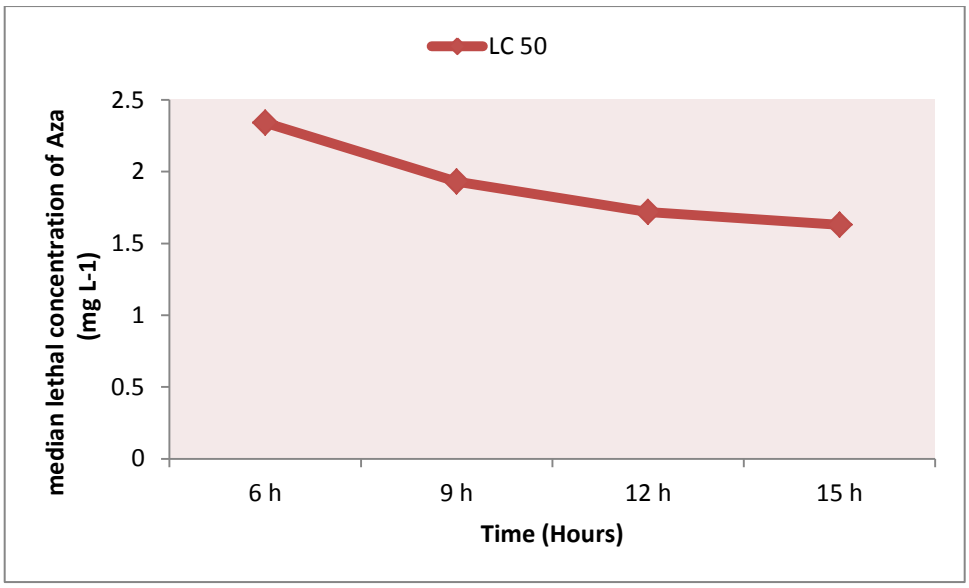


Fig 20. Median lethal concentration (LC_{50}) of allicin (EC 100%) against goldfish at three hours interval upto 15 hours.

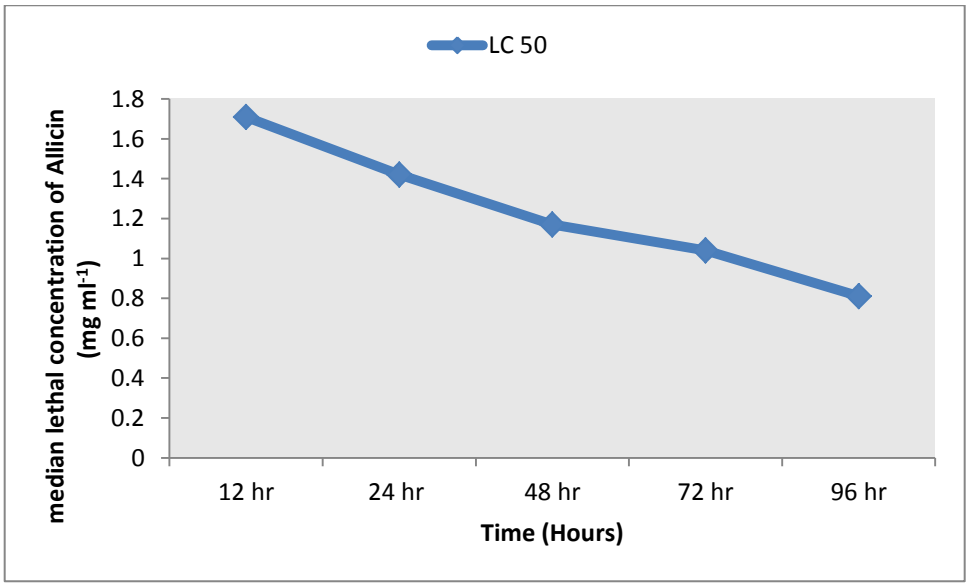


Fig 21. median lethal concentration (LC_{50}) of allicin (EC 100%) against goldfish at twenty four hours interval upto 96 hours.

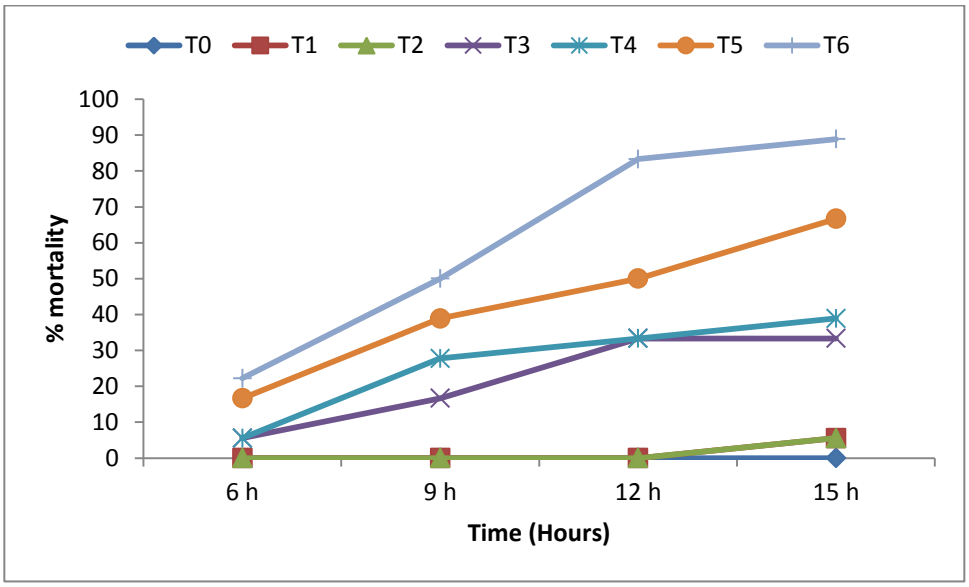


Fig 22. Cumulative percentage mortality of fish with respect to time when exposed to different concentrations of allicin (EC 100%) upto 15 hours

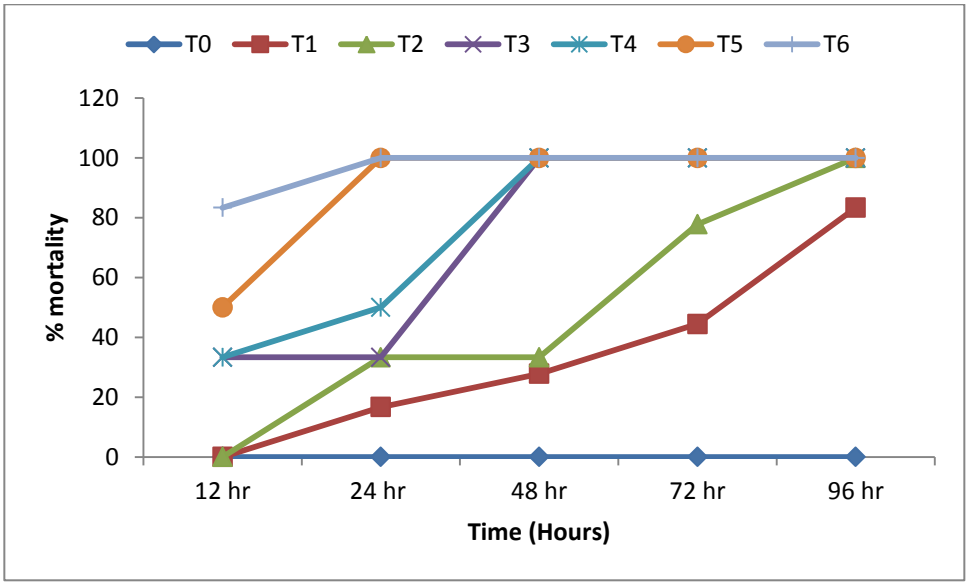


Fig 23. Cumulative percentage mortality of fish with respect to time when exposed to different concentrations of allicin (EC 100%) upto 96 hours

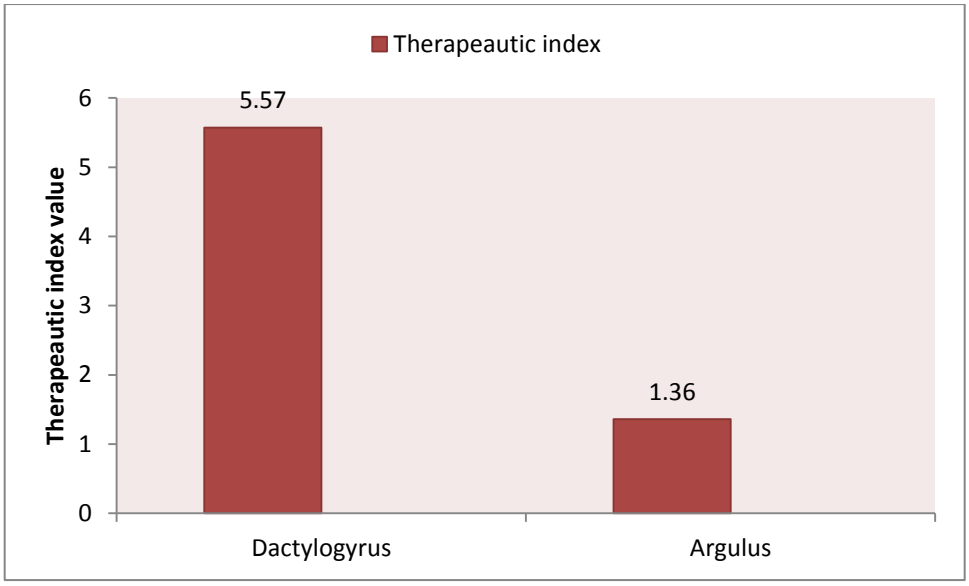


Fig 24. Therapeautic index value for allicin (EC 100%) against common goldfish ectoparasites

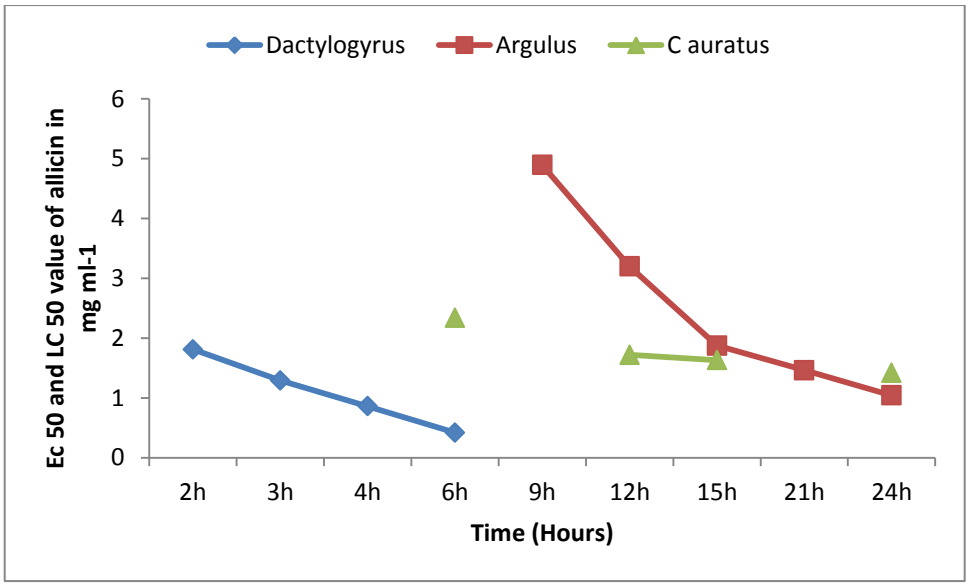


Fig 25. Comparative graph showing the median effective concentration (EC_{50}) values of allicin (100%) against common goldfish ectoparasites and median lethal concentration (LC_{50}) values against goldfish to understand the use of allicin for *in vivo* treatment of ectoparasites in goldfish.

5. DISCUSSION

5.1 Prevalence of Ectoparasites on Fish

Ectoparasites mostly *Argulus* spp., *Ichthyophthirius* sp. and both *Dactylogyrus* and *Gyrodactylus spp*. (monogenean) infesting goldfish are reported to cause great damage to the host and also affects its global trade (Noaman *et al.,* 2010; Chanda *et al.,* 2011; Iqbal *et al.,* 2014; Khodadadi *et al.,* 2013; Macchioni *et al.*, 2015). Among the ornamental fish being imported in Pakistan, it was found that 69.10% fish were infested with ectoparasites with the highest prevalence in goldfish (75%) and molly (52%) as reported by (Iqbal *et al.,* 2014). Chanda *et al.* (2011) reported the prevalence of 77.36% ectoparasites with *Ichthyophthirius* sp. showing the highest prevalence of 65% in goldfish from three districts of West Bengal in a sample size of 159 fish. A study on ecology of metazoan parasites in 13 freshwater fish species in kerala revealed that out of a sample size of 2,634 fish, 62.5% were infested with metazoan parasites with the highest prevalence of digenean followed by nematoda and monogenean parasites (Beevi and Radhakrishnan, 2012). A prevalence of 60% ciliate ectoparasites and 48% crustacean ectoparasites was reported from *Labeo rohita* and *Schizothorax niger* from Jammu and Kashmir (Dar *et al.,* 2014). Monir *et al.,* (2015) reported high prevalence of 94.54% parasites in *L. rohita* and lot prevalence of 72.68% from *Catla catla* cultured in three districts of Bangladesh, Further they concluded that parasitic diseases affected the study areas mainly by causing fish mortality (11%), increasing cost of chemicals (11%) and affecting the growth of fish (65%). Most of the study reveals that cyprinid group of fishes are vulnerable to parasitic infestations and as most of the fishes from this group are cultured worldwide as foodfish or ornamental fish, ecology of parasites associated with cyprinid fish must be understood.

Goldfish is an important ornamental fish, easily available and highly demanded in the market, is susceptible to infestation by ectoparasites and is also used as a biological model for edible carps (Sokolowska *et al.* 1984) and the results of the present study on prevalence of ectoparasites infesting three ornamental fish species shows the highest prevalence of 56.6% in goldfish, therefore goldfish was chosen as the experimental fish.

5.2 Physico-Chemical Parameters of Water

The well being of fish depends on the physico-chemical parameters of the water that can either keep the fish healthy or provide opportunity to different fish pathogens to multiply. Ornamental fish are kept in captivity, hence they have the ability to live under a wide range of environmental condition (Chapman, 2000). All the physico-chemical parameters of water measured during the course of experiment such as temperature, pH, dissolved oxygen, free carbondioxide and alkalinity were found to be within the optimum range of requirements for goldfish.

5.2.1 Temperature

One of the most important environmental variables is temperature (Alzieu, 1989). It plays an important role in regulating the metabolism of animals and the optimum range varies from species to species. Goldfish can thrive well at temperature range of 18-38 ^{0}C (Alzieu, 1989) and the present range of recorded temperature 23.5-31.5 ^{0}C is well within the limit of goldfish.

5.2.2 pH

The pH range was 7.3-8.1 in the experimental tanks which lies within the optimum limit 6.5 to 9.0 as suggested by (Swingle, 1967).

5.2.3 Dissolved oxygen

For better survival and growth of ornamental fish, the DO ought to be maintained above 5 mg L^{-1} (Chapman, 2000). The dissolved oxygen level varies in water due to various factors influencing it such as temperature, biomass density, metabolic rate etc. The dissolved oxygen recorded from experimental tanks were in the range 6.4-7.4 mg L^{-1} which is assumed to lie within the optimum limit of cyprinids 6-7 mg L^{-1} as suggested by (Huet, 1971).

5.2.4 Free carbondioxide

The carbondioxide was not detected at any time in the experimental tanks and also did not affect the growth and survivability of the experimental fish. This may be due to low biomass, continuous aeration and water exchange.

5.2.5 Alkalinity

Water with minimum alkalinity of 20-40 mg L^{-1} $CaCO_3$ is considered optimum for both cold and warm water fish (Stickney, 1994) but alkalinity in range 100-200 mg L^{-1} provides additional buffering capacity required to eliminate wide pH fluctuation in the culture system and provide carbon for biological productivity (Wedemyer, 1996). The alkalinity recorded in the present study was in range 182 – 208 mg L^{-1}.

5.3 Artificial Infection of Goldfish with *Argulus* spp.

Artificial infection of goldfish was carried out for 5 months and prominent infestation of *Argulus* was observed after 3 weeks of challenge. The infection was passaged either through cohabitation method or by transferring substrates containing *Argulus* eggs into a different tank containing healthy goldfish. The results of the present study shows an average intensity of 40-50 small *Argulus* per goldfish with some fishes being infected with upto 200 parasites. Similar results, showing an increase in the population of *Argulus* in goldfish kept in cohabitation with *Argulus* infested fish was reported by Kumar *et al.* (2012b) with a moderate infection of 15-20 *Argulus* per fish. According to a study by kone *et al.* (2014), healthy nile tilapia kept in cohabitation with *Argulus* infested fish for six months showed a mean intensity of 9.9-31.8 parasite per fish causing behavioural changes in fish and mortality upto 59% thereby concluding that *Argulus* was responsible for mortality of nile tilapia cultured in Cote d'lvoire (Forlenza *et al.,* 2008) collected adult *A. japonicus* from stock carp, hatched the eggs and exposed individual fish to 150 larval lice in a beaker. Silt free hard substrata like irregular stones, plastic boards, wooden sticks, rocks, plant stems and concrete are used by *Argulus* spp. for laying eggs (Hakalahti *et al.,* 2004, Walker *et al.,* 2004 and Sahoo *et al.,* 2013). Similar results were observed in the present study where *Argulus* spp layed eggs

on stem of hydrilla, stone, stone slab, glass walls of aquaria, wooden sticks and aeration pipes. Rohu with heavy infestation with *Argulus* spp of 200-300 early juvenile parasites per fish were used by (Saurabh *et al.,* 2010) for conducting artificial infection of *Argulus* through cohabitation method where fin clips containing early juvenile *Argulus* were added to tanks containing ten healthy rohu and the intensity of parasite was found to be less than 10 to more than 25 parasites per fish after 12 days. At 28^0 C most *Argulus* were found on the flank, caudal fin and pectoral fin of carp while at lower temperatures, large parasites (2.8mm) were found on the surface of operculum (Schluter, 1978). In case of chronic infection with *Argulus*, fish shows symptoms of listlessness, frayed fins, opaque skin and haemorrhagic spots (Stammer, 1959).

In the present study Argulus associated haemorrhagic sites and fish mortality was observed during the experimental artificial challenge study.

5.4 Artificial Infection of Goldfish with *Ichthyophthirius multifiliis*

In the present study, healthy goldfish were cohabitated with *I. multifiliis* infected goldfish in an aquarium (5:1). The mature parasites dislodged from the infected fish, encysted into tomonts and finally theronts were released in water that infected the other fish in the aquarium. Prominent infestation of the parasite was observed from 7^{th} day onwards after the artificial challenge. Sahandi *et al.* (2012) conducted artificial infection of *I. multifiliis* in sail fin molly by collecting tomonts from heavily infected fish and keeping it in a beaker containing 1000 ml water for production of theronts which was used to infect healthy fish. Five heavily infected rainbow trout with *I. multifiliis* were kept in cohabitation with 30 healthy rainbow trouts (1:5) in the same tank and infection was observed from 3^{rd} day onwards after theronts were produced from the trophonts (Jorgensen and Buchmann, 2007) which shows similarity with the present study. Ling *et al.* (2010) used 1000, 2000, 4000, 8000 and 16000 infective theronts per goldfish to get *I.multifiliis* infection and the results showed that exposure to 4000 theronts per fish at 22 ± 2 ^{0}C was effective in causing 100% prevalence of the parasite. It is reported that encysted tomonts at 20-25 ^{0}C produces theronts in 24 h which is capable of growing into mature trophonts on healthy fish in 5-6 days. The temperature of water in the

present artificial challenge period was 23.5 ^{0}C to 27.4 ^{0}C which was favourable for the parasite. (Schlenk *et al.,* 1998) maintained healthy channel catfish fingerling with *I. multifiliis* infested fish at 18^0 C in 40 L aquarium tank until all the fish were infected and also determined that 2000 theronts per litre of water was effective in 100% infestation of the parasite at the same temperature. (Yi *et al.,* 2012) maintained *I. multifiliis* infestation in goldfish by passaging the parasite using a combination of healthy and infected fish at 20 ± 2^{0}C.

In the present study *I. multifiliis* infection was maintained at temperature 23.5 ^{0}C to 27.4 ^{0}C but as the water temperature started increasing, fish mortality due to heavy infestation of the parasite was observed and finally the infection collapsed at temperature above 28 ^{0}C that shows temperature dependent infection of the parasite.

5.5 *In vitro* Study of Azadirachtin (EC 21.5%) and Allicin (EC 100%) Against Common Ectoparasites Infesting Goldfish

Azadirachtin and allicin are herbal bioactive molecules from Neem and garlic respectively. Azadirachtin belongs to the triterpenoid groups of molecules which is highly oxygenated with fourteen oxygen atoms in five ester groups, three free hydroxy groups and a free dihydrofuran ring (Butterworth *et al.,* 1972). Allicin consists of sulphur-sulphur bond which allows it to react with thiol groups of enzymes (Ankri *et al.,* 1999). In the present study, azadirachtin of 21.5% purity and allicin of 100% purity were evaluated against common ectoparasites of goldfish.

5.5.1 *In vitro* antiparasitic effect of azadirachtin and allicin against *Argulus* spp.

Kumar *et al.* (2012b) reported that azadirachtin of 25% purity showed 100% antiparasitic efficacy against *Argulus* spp. under *in vitro* conditions at concentrations of 20 and 15 mg L^{-1} respectively in 2.5 and 3h. It was also reported in the same study that *Argulus* spp. exposed to concentrations of 1, 5, 10, 15 and 20 mg L^{-1} of azadirachtin caused 30%, 45%, 75%, 85% and 100% mortality of the parasite respectively in 3 h showing a dose dependent mortality of the parasite. Similar results were obtained in the

present *in vitro* study where dose dependent mortality of *Argulus* spp. was observed on exposure to azadirachtin and allicin. The percentage mortality of *Argulus* spp. exposed to azadirachtin concentrations of 25, 50, 75, 100 and 125 mg L^{-1} was in increasing order of 50%, 66.67%, 77.58%, 100% and 100% respectively in 12 h for adult *Argulus* and 50%, 66.67%, 83.33%, 100% and 100% respectively in 12 h for juvenile *Argulus* which shows similarity to the trend of mortality observed by (Kumar *et al.,* 2012b). The *in vitro* antiparasitic efficacy of azadirachtin was found to be 100% against adult and juvenile *Argulus* at 100 and 125 mg L^{-1} concentrations in 15 and 12 h respectively that shows variations from the previous study by Kumar *et al.* (2012b) in terms of dose of compound and time of mortality of parasite. The difference mey be attributed to the variation in the purity of herbal product, solvent used for dissolution of the molecule (DMSO vs methanol), stock of *Argulus* and their response to chemical product, abiotic fators that inhibit/ promote the efficacy of HBMs (pharmacodynamics of chemicals) and lastly the production and purification procedure used for the synthesis of HBMS.

Allicin used at concentrations of 2, 2.5, 3, 3.5 and 4 mg ml^{-1} caused 77.78%, 77.78%, 88.89%, 94.44% and 100% mortality of *Argulus* in 18h under *in vitro* conditions. These findings are in accordance with the reports of Kumar *et al.* (2012a) where positive correlation of *Argulus* mortality at different dose of piperine solution was observed. The *in vitro* antiparasitic efficacy of allicin against *Argulus* was 100% at 4 and 3.5 mg ml^{-1} in 18 and 21h respectively which shows wide variation as compared to (Kumar *et al.,* 2012a) where antiparasitic efficacy of piperine was found to be 100% at 9 mg L^{-1} in 3h. (Kone *et al.,* 2013) reported the LC_{50} of crude extract of African traditional plant *Ocimum gratissimum* to be 197.66 mg L^{-1} against *Argulus* sp. under *in vitro* conditions while the present study reveals a better EC_{50} of azadirachtin against adult and juvenile *Argulus* being 39.04 mg L^{-1} and 37.78 mg L^{-1} in 15h respectively.. The results of *in vitro* effect of Neem leaf extract on adult *Argulus* showed that the LC_{50} of the parasite in 24h was 259.42 mg L^{-1} (Banerjee *et al.,* 2014) which is higher than the EC_{50} obtained in the present study. (Hakalahti *et al.,* 2008) found in a study that small juvenile *Argulus* (<0.8mm) and adult *Argulus* (10.3mm), when treated with potassium permanganate showed less variation in the death rate when compared to juvenile *Argulus* (2.3mm). Similar finding was observed in treatment of adult and juvenile

Argulus with azadirachtin where the variation in percentage mortality is mereand not significant.

The present *in vitro* study revealed that azadirachtin (a bioactive molecule of neem) is highly potent to *Argulus* spp. at lower concentrations compared to allicin (a bioactive molecule of garlic) which are in accordance with the results of authors mentioned above.

5.5.2 Effect of azadirachtin on *Argulus* eggs under *in vitro* conditions

Argulus has a direct life cycle consisting of laying eggs on suitable substrates by female parasites and hatching of parasitic metanauplius from these eggs. An experiment was carried out to check whether azadirachtin was able to cause disturbance or arrest the hatching of *Argulus* eggs under *in vitro* conditions. Results of the present study shows that 100% hatching of *Argulus* eggs were observed in the control group and 25 and 50 mg L^{-1} azadirachtin solution in 14 days at temperature 29.5-31.5 ^{0}C with the first hatching observed on 8th day from the date of start of experiment. These observations goes in accordance with (Fryer, 1982) stating eggs of *Argulus foliaceus* hatch after just 8 days at 26^{0} C. On treatment with formalin (120 ml L^{-1}) for 24h, it was found that there was significant mortality of *A. coregonii* eggs (Hakalahti *et al.,* 2008). The results of our experiment shows significant reduction in hatching of eggs at 150 and 175 mg L^{-1} concentrations of azadirachtin that shows almost equally similar results compared to formalin. Treatment of *Argulus* eggs with Neem leaf extracts at concentrations of 120 and 250 mg L^{-1} for 70 min showed coagulation of yolk and reduction in percentage hatching of eggs (Banerjee *et al.,* 2014) which supports the results obtained in the present study. Tobacco leaf dust containing nicotine was found to be ineffective against *Argulus* eggs submerged in 8ppm of nicotine solution for 18 days (Banerjee *et al.,* 2013). Our study shows similar results at lower concentrations of azadirachtin (below 100mg L^{-1}) where hatching of eggs was not arrested. 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 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.

The present study is first of its kind where *Argulus* eggs are bath treated with azadirachtin under *in vitro* conditions. The results obtained are satisfying the results obtained by authors discussed above. The findings can be useful in treating eggs present in ponds or aquariums before stocking of fish, yet study regarding revival of eggs on transferring in water after treatment with azadirachtin is to be done.

5.5.3 *In vitro* antiparasitic effect of azadirachtin against theronts of *I. multifiliis*

To prevent the spread and infection of *I. multifiliis*, the most important stage of the life cycle to be targeted are the free living stages, theronts and tomonts (Heinecke and Buchmann, 2009). The present *in vitro* study was also based on breaking the life cycle of ich by evaluating the effect of azadirachtin on *I. multifiliis* theronts. Psoralidin, an active compound from the plant *Psoralea corylifolia* was able to kill 300 theronts at 0.8 mg L^{-1} concentration or more in a exposure time of 4h, protomonts at 0.9 mg L^{-1} and encysted tomonts at 1.2 mg L^{-1} concentration in 6h respectively thereby showing better antiparasitic effect at higher concentrations (Song *et al.,* 2015). The results of the present study also shows the same trend of mortality of theronts against different concentrations of azadirachtin where 100% mortality of theronts was observed in 6h at 47.61 mg L^{-1} concentration of azadirachtin. Ling *et al.* (2010) reported that *I. multifiliis* theronts exposed to 4.8 mg L^{-1} of potassium ferrate(VI) for 4h showed 100% mortality with the LC_{50} being 1.71 mg L^{-1}. Aqueous extract of *Capsicum frutescens* was found to kill 70% theronts at concentrations of 1:32 and 1:64 (Volume of stock solution: volume of total solution) in 4h (Ling *et al.,* 2012). Similarly, Yi *et al.* (2012) reported that out of 30 medicinal plants screened for treatment against *I. multifiliis,* methanol extracts of *Magnolia officinalis* and *Sophora alopecuroides* were found to be most effective against theronts with 4-h LC_{50} values estimated to be 2.45 and 3.43 mg L^{-1} respectively. The results of the present study reveals the EC_{50} of azadirachtin against theronts to be 38.42 mg L^{-1} in 6h, thereby showing that it posseses efficacy against theronts. (Sahandi *et al.,* 2012) reported that 0.1g L^{-1} and 0.4g L^{-1} *Allium sativum* and *Matricaria*

chamomilla extract respectively were able to treat *I. multifiliis* infection in sail fin molly in 5 days. Ekanem *et al.* (2004) reported that crude methanolic extract of leaves of *Mucuna pruriens* and the petroleum-ether extract of seeds of *Carica papaya* were able to kill 100% *I. multifiliis* under I condition in 6h at concentrations 150 and 200 mg L^{-1} respectively. The present findings of azadirachtin against theronts shows better results compared to *Mucuna pruriens* and *Carica papaya*.

5.5.4 *In vitro* antiparasitic effect of azadirachtin and allicin against *Dactylogyrus* sp.

In vitro test of azadirachtin against *Dactylogyrus* sp. showed that 100% efficacy was obtained at 30 mg L^{-1} in 3h while the same efficacy was obtained by allicin at 4 mg ml^{-1} concentration in 2h which shows azadirachtin being more potent towards *Dactylogyrus*. (Malheiros *et al.,* 2016) reported that essential oil of *Mentha piperita* was effective against *D. cycloancistrium* and *D. cycloancistrioides* at 80 mg L^{-1} in 5h. The 96h EC_{50} of ethyl acetate extract of *Euphorbia fischeriana* was 13.65 mg L^{-1} against *D. vastator* (Zhang *et al.,* 2014) which supports the results of present study where EC50 of azadirachtin against *Dactylogyrus* was estimated to be 6.08 mg L^{-1} in 4h showing better results than *E. fischeriana.* The antiparasitic effect of ethanolic and aqueous ginger extract were found to be efficient at 75 and 200 mg ml^{-1} towards *Gyrodactylus turnbulli* infesting guppy at 65.6 ± 2.8 and 1.8 ± 0.2 min (Levy *et al.,* 2015) which shows higher concentrations compared to allicin which was effective at 4 mg ml^{-1} against *Dactylogyrus* in 2h. Aqueous garlic extract was able to kill *G. turnbulli* infecting guppy under *in vitro* conditions at cocentrations between 7.5 and 30ml L^{-1} showing positive correlation of parasite mortality with time. Similar results were obtained using azadirachtin and allicin against *Dactylogyrus* sp where time taken for parasite mortality reduced with the increase in the concentration of the compound in treatment. Chinese freeze dried garlic powder and freeze dried garlic flakes were found to be effective against *G. turnbulli* infecting guppy at concentrations less than 1 mg ml^{-1} by reducing the parasites survival time from 13h to less than 1h (Schelkle *et al.,* 2013). Wang *et al.* (2008) reported two active compounds osthol and isopimpinellin to be 100% effective against *D. intermedius* at 1.6 and 9.5 mg L^{-1} concentrations respectively. Garlic oil was

reported to be effective in treating gyrodactylosis and trichodinosis in *Oreochromis nilotica* fries at concentrations of 3 ppt thereby showing antiparasitic efficacy in *in vivo* bath treatment for 4h (El-Galil and Aboelhadid, 2012). Azadirachtin and allicin are more effective against *Dactylogyrus* compared to *Argulus* and *theronts* which may be attributed to the protection of theronts by rows of beating cilia (Matthews *et al.,* 1996), *Argulus* bears a hard exoskeleton for protection (Moller, 2009) while *Dactylogyrus* lacks any such protective covering (Fried, 1991) which makes it more susceptible to the evaluated compounds.

The *in vitro* results of the selected HBMs against *Dactylogyrus* obtained by us shows azadirachtin to be more efficacious than allicin while both the compound satisfy the results obtained by different authors in terms of dose dependent mortality and dose of the compound used for treatment.

5.6 Acute toxicity of azadirachtin (EC 21.5%) and allicin (EC 100%) against goldfish

The results of acute toxicity tests of azadirachtin for goldfish expressed in terms of LC_{50} values were 78.36, 59.47, 25.07 and 20.48 mg L^{-1} for 3, 6, 12 and 15 h, respectively. The result of median lethal concentration LC_{50} for goldfish reveals the gradual decrease in dose with increase in time. Similar results were reported by Kumar *et al.* (2012b) as LC_{50} values of Aza (EC25%) for goldfish were 98.645, 88.793 and 82.115 mg L^{-1} for 48, 72 and 96 h, respectively. Allicin also showed the same trend with its LC_{50} value for 9, 12 and 15 h being 1.93, 1.72 and 1.63 mg ml^{-1}. The results show that allicin is more safe for goldfish at higher cncentrations than azadirachtin. A similar result was also reported by Kumar *et al.* (2012a) where LC_{50} values of piperine for goldfish was found to be 60.05, 56.33 and 52.64 mg L^{-1} for 48, 72 and 96h respectively. Previous study shows the LC50 value of azadirachtin against *Heteropneustes fossilis* for 48, 72 and 96h to be 80.69, 58.57 and 52.35 mg L^{-1} respectively concluding the fish to be a hardy species (Kumar et al., 2011). The 96h LC_{50} value of azadirachtin containing product, nimbecidine and neem gold were found to be as low as 0.135 mg L^{-1} for nimbecidine and 0.525 mg L^{-1} for neem gold for a freshwater loach

Lepidocephalichthys guntea (Mondal *et al.,* 2007) which shows a lesser value compared to the results of azadirachtin obtained in the present study. Another study shows the LC_{50} of neem leaf extract for 24h as high as 4.8 g L^{-1} for *Prochilodus lineatus* (Winkaler *et al.,* 2007). In a study by Syngai *et al.* (2016) it was found that the 96h LC_{50} of aqueous garlic extract for *Cyprinus carpio was* 253.19 mgL^{-1} showing less value than the 96h LC_{50} value of allicin against goldfish in the present study which is 0.81 mg ml^{-1}. The results of LC_{50} shows variation with the compound being used and fish species under study.

The results of the present study shows safety of goldfish at lower concentration of azadirachtin and at comparatively higher concentration of allicin. The results fits in with some of the results of the authors discussed above while it varies in some cases. The reason for this variation can be attributed to the purity of the compound, the inherent tolerat potential of the host and source of herbal bioactive molecules.

5.7 Therapeautic Index of Azadirachtin and Allicin

Under *in vitro* conditions, the estimated therapeautic index shows safe dose of azadirachtin for *Dactylogyrus* (5.33) and theronts (1.67) but critical for juvenile and adult *Argulus* (0.69 and 0.65 respectively). Similarly, the therapeautic index of allicin shows safe dose for *Dactylogyrus* (5.57) and critical for *Argulus* (1.36). The results shows that both the compounds are more effective against *Dactylogyrus*. The mortality of *Argulus* parasite in azadirachtin solution *in vitro* shows similarity with (Kumar *et al.,* 2012b) but disagreed in terms of therapeautic index which was 4.10 in case of Aza (EC 25%). Though, the therapeautic index of azadirachtin and allicin against *Dactylogyrus* are in accordance. Similarly, the therapeautic index of two compounds osthol and isopipinellin from *Fructus cnidii* was found to be 8.42 and 1.52 respectively against *D. intermedius* showing that osthol was a better choice than isopimpinellin, despite both can be used for controlling *D. intermedius* in aquaculture (Wang *et al.,* 2008). The therapeautic index of piperine was reported to be 5.8 against *Argulus* infesting goldfish (Kumar *et al.,* 2012a).

The results of the present study brings to the conclusion that both the herbal bioactive molecules (HBMs) were found to be effective against ectoparasites infesting goldfish under *in vitro* conditions but there was variation among their antiparasitic efficacy. Azadirachtin of 21.5% purity was able to cause mortality of parasites under *in vitro* conditions at much lower concentrations compared to Allicin (EC 100%). There was a difference in the EC_{50} values of azadirachtin against different ectoparasites which may be due to the physiology and anatomy of the parasites, since they belong to different taxa and phyla. Allicin also showed difference in the EC_{50} values between *Dactylogyrus* and *Argulus spp.* which can be attributed to the same reason mentioned. The therapeautic index shows that azadirachtin and allicin can be safe for treating *Dactylogyrus* infection in goldfish and to a lesser extent azadirachtin is also safe for treating *I.multifiliis* theronts of goldfish because of its low EC_{50} value against the parasite. In neither case any of the two compounds were found to be safe for treating *Argulus* infesting goldfish due to low therapeautic index. Theronts stage of *I. multifiliis* are free swimming,therefore azadirachtin can be used for treating the water containing theronts in case of Ich outbreak in goldfish. Since, the requirement of allicin used in the present study for treating *Argulus* and *Dactylogyrus* is very high and also as it is a costly compound, it may not be feasible to be used in ornamental fish industry for controlling ectoparasites.

Thus, it can be inferred from the present study that on the basis of cost effectiveness and minimal use of resources, azadirachtin (EC 21.5%) will be considered as a potent herbal bioactive molecule possessing broad spectrum *in vitro* antiparasitic efficacy against goldfish ectoparasites that can be safe for treating *Dactylogyrus* and *theronts* infection in goldfish at concentrations below 20.48 mg L^{-1}.. The study also opens new avenues about azadirachtin (EC 21.5%) and allicin (EC 100%) for future study such as mechanism of killing of ectoparasites, reasons for variation between the efficacy of the two compounds, *in vivo* study, effective mode of administration of the compounds for obtaining better results, effect on different fish host and withdrawl time of the compounds. On the whole, use of herbal bioactive compounds may aid to the sustainable aquaculture and ornamental fish rearing.

SUMMARY

Aquaculture industry is expanding rapidly and ornamental fish production is also gaining importance in recent years. Every development carries some bottlenecks and one of the major problems in aquaculture industry is the occurrence of different types of viral, bacterial and parasitic diseases. A bewildering array of parasites are present in the aquatic environment, among which ectoparasites are very important as they cause direct damage to the host by causing mortality, reduced growth, affecting appearance of the fish and indirectly by acting as vectors for other pathogens and reduction in its economic and aesthetic value. Ectoparasites affect ornamental fish industry more severely in terms of aesthetic point of view. Macro and meso-ectoparsites are a great menace in carp aquaculture system including ornamental fish industry. It remains attached on the body surface of the host and adult parasites are easily visible through naked eye. They cause irritation in fish due to their movement on the body surface and their feeding habits. Metazoan ectoparasites (Argulids and monogenea) remain anchored to the body surface and gill filament and draw nutrition from them, thereby causing anaemia, restlessness, gill necrosis and mortality in fish. *Ichthyophthirius multifiliis* is the largest protozoan parasite infesting fish and is known for its rapid multiplication rate and associated mortality of fish. There have been little promising treatments for ectoparasites and most of the chemicals and synthetic drugs used in the past have been banned due to their ill effects on the host and environment. An efficient alternative that is promising, cost effective and eco-friendly to this is phytotherapy which makes use of available plant extracts or their active molecules as a therapeautic agent. Neem and garlic are known for their medicinal purpose throughout the world with their important bioactive molecule being azadirachtin and allicin respectively. The present study aims to evaluate the antiparasitic efficacy of selected herbal bioactive molecules (azadirachtin and allicin) against particular ectoparasites of freshwater ornamental fish.

The ornamental fish for the experimental purpose was selected after conducting a preliminary screening of three ornamental fish (goldfish, koi carp and molly) for the presence of ectoparasites from three different locations in Mumbai and fish showing the highest prevalence of parasite was chosen for the experiment. Since, goldfish was

found to be more infested with ectoparasites. Goldfish were artificially infected with *Argulus* and *I. multifiliis* and then azadirachtin and allicin were tested for their antiparasitic efficacy against these parasites as well as *Dactylogyrus* sp.

The antiparasitic activity of azadirachtin under *in vitro* condition was performed from range 1 to 175 mg L^{-1} against different stages of selected ectoparasites. The 100% *in vitro* antiparasitic efficacy was observed in T_4 (100 mg L^{-1}) and T_5 (125 mg L^{-1}) treatment groups in 15 and 12h respectively for both adult and juvenile *Argulus*. The EC_{50} of azadirachtin against *I. multifiliis* theronts was found to be 34.42, 20.94 and 11.83 mg L^{-1} in 6, 9 and 12h respectively and the 100% *in vitro* antiparasitic efficacy was observed in 6h in T_5 (47.61 mg L^{-1}) treatment group. The EC_{50} of azadirachtin against *Dactylogyrus* was 21.65, 14.70 and 6.08 mg L^{-1} respectively in 2, 3 and 4h with 100% *in vitro* antiparasitic efficacy in 3 and 4h at T_7 (30 mg L^{-1}) and T_6 (25 mg L^{-1}) treatment groups respectively. Azadirachtin also interfered with the hatching of *Argulus* eggs with 50% reduction in hatching of eggs kept in T_6 (150 mg L^{-1}) and T_7 (175 mg L^{-1}) treatment groups. It also affected the survival of hatched nauplius that survived 36-48h in control groups but less than 6h in treatments above 50 mg L^{-1}. The results showed that azadirachtin was potent to common ectoparasites of goldfish at lower concentrations under *in vitro* conditions.

Similarly, antiparasitic activity of allicin under *in vitro* condition was performed at 2, 2.5, 3, 3.5 and 4 mg ml^{-1} for *Argulus* spp.; 1, 1.5, 2, 2.5, 3, 3.5, 4 mg ml^{-1} for *Dactylogyrus* sp. The results of EC_{50} against *Argulus* was found to be 4.89, 3.19 and 1.87 mg ml^{-1} for 9, 12 and 15h respectively. The 100% *in vitro* antiparasitic efficacy of allicin against *Argulus* was observed in 18h at T_5 (4 mg ml^{-1}) concentration. Allicin showed 100% *in vitro* antiparasitic efficacy against *Dactylogyrus* in 2h in treatment group T_7 (4 mg ml^{-1}) and 3h in treatment groups T_5 (3 mg ml^{-1}) and T_6 (3.5 mg ml^{-1}) respectively. The EC_{50} against *Dactylogyrus* was 1.8, 1.28 and 0.85 mg ml^{-1} respectively in 2, 3 and 4h. The results show that allicin is also effective against goldfish ectoparasites but at a much higher concentration than azadirachtin.

The LC_{50} of both the herbal bioactive molecules were conducted for goldfish to ensure the safety of the host for *in vivo* treatment. The 9, 12 and 15h estimated LC_{50} was found to be 39.58, 25.07 and 20.48 mg L^{-1} for azadirachtin and 1.93, 1.72 and 1.63 mg ml^{-1} for allicin respectively showing more safety of allicin at higher concentrations compared to azadirachtin.

Using the LC_{50} and EC_{50} values of azadirachtin and allicin obtained through probit analysis, the therapeautic index were estimated. The therapeautic index of azadirachtin against adult *Argulus,* juvenile *Argulus* (for nine hours), theronts and *Dactylogyrus* (for three hours) was estimated to be 0.65, 0.69, 1.67 and 5.33 respectively which shows that azadirachtin can be used for treating *Dactylogyrus* and *I. multifiliis* infection in goldfish but is critical to be used against *Argulus* infestations in goldfish. Similarly the therapeautic index of allicin against *Argulus* and *Dactylogyrus* was estimated to be 5.57 and 1.36 respectively for twenty four hours which shows that it can also be used for treating *Dactylogyrus* infestation in goldfish but is critical against *Argulus* infestation in goldfish.

It was inferred that since azadirachtin of purity 21.5 % was effective in less time and at low concentration than allicin (100%), it had better broad spectrum *in vitro* antiparasitic efficacy against common ectoparasites infesting goldfish. Azadirachtin at concentrations of less than 20.48 mg L^{-1} was found to be safe for goldfish for fifteen hours that can be used for treating *Dactylogyrus* and ich infection. Similarly allicin can be used for treating *Dactylogyrus* in goldfish below 1.63 mg ml^{-1}. Azadirachtin can also be used for treating *Argulus* eggs in aquarium tanks before stocking fish at concentrations of 175mg L^{-1}. From cost effective point of view azadirachtin was found to be a potent antiparasitic agent under *in vitro* conditions.

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ABBREVIATIONS

%	:	Percentage
µg	:	Microgram
^0C	:	Degree Centigrade
A.E.	:	Antiparasitic Efficacy
APHA	:	American Public Health Association
Aza	:	Azadirachtin
cm	:	Centimetre
CO_2	:	Carbon Dioxide
DO	:	Dissolved Oxygen
EC	:	Effective Concentration
EC_{50}	:	Median Effective Concentration
g	:	Gram
h	:	Hour
HBMs	:	Herbal Bioactive Molecules
L	:	Litre
LC_{50}	:	Median Lethal Concentration
mg	:	Milligram
mg L^{-1}	:	Milligram per litre
mg ml^{-1}	:	Milligram per millilitre
min	:	Minutes
ml	:	MIllilitre
SPSS	:	Statistical Package for Social Science
T	:	Treatments