Aquaculture 473 (2017) 181–190

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Phytochemical screening for identification of bioactive compound and antiprotozoan activity of fresh garlic bulb over trichodinid ciliates affecting ornamental goldfish

Mandira Saha, P.K. Bandyopadhyay *

Parasitology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India

ARTICLE INFO

Article history:
Received 21 December 2016
Received in revised form 3 February 2017
Accepted 5 February 2017
Available online 10 February 2017

Keywords:
Trichodiniasis
Phytochemicals screening
Antiprotozoan activity
Allium sativum (Garlic)
GC-MS analysis
Bioactive compound
Ornamental fish

ABSTRACT

Ornamental fish culture is an economically important business of our country and contribute in the socioeconomic upliftment of the backward community with a little investment. However, parasitic outbreaks cause increased mortality, which in turn results in high economic loss in this industry. Trichodinids are known to be a major problem in fish farms causing serious damage, particularly under cultured condition of gold fish. The wide variety of chemicals used for treating trichodiniasis not only make the environment unfavorable for aquaculture but the pathogen also becomes resistant towards them over time. So, the chemicals should be used more rationally to prevent the protozoa from becoming resistant towards them and moreover there is an urgent requirement of alternative therapeutic strategies to control this protozoon mediated damage of the ornamental fishes. Hence, the present study has been designed to identify a non-chemotherapeutic agent for disease treatment in aquaculture which might be beneficial for the industry. Garlic is popularly known to be rich in a variety of secondary metabolites owing the ability to synthesize many different substances that has been widely exploited by the local community for its medicinal value, which led us to test it as a therapeutic agent for trichodiniasis in goldfish. After determining its toxicity level (LC_{50} value-29.79 mg/L) 15 mg/L ethanolic extract of garlic was found to reduce the trichodinids burden of goldfish significantly ($P < 0.01$) within 4 days only which clearly demonstrates the antiprotozoan activity of fresh garlic bulb. Further the bioactive components of garlic responsible for its antitrichodinid activity were identified using GC-MS.

1. Introduction

Ectoparasitic ciliates are considered as the major problem for ornamental fish farming being one of the major factors responsible for the devastating economic losses of aquaculture through mortality and decreased growth rate of fish (Silva and Turchini, 2008). The highly intensified systems in ornamental fish farms with high temperature and organic contents use to accelerate the life cycles of the parasites (Basson and Van, 1994; Hassan, 1999; Davis et al., 2002;). They are regarded as a main cause of fish mortality (Ramadan et al., 1995; Abdel-Meguid, 2001). Therefore, to inhibit this problem some necessary actions are needed to be taken. The development of disinfection agents to treat protozoan infestation is one of the most fascinating stories in the history of aquatic animal health. Trichodinid are the most common ciliate ectoparasites, which are the causative agents of a very serious disease known as trichodiniasis in gold fish affecting their skin and gills (Iguchi et al., 2003). The infected fishes become lethargic, generate excessive mucus and become off-feed which eventually leads to death in most of the cases.

Current strategies for controlling trichodiniasis in aquaculture primarily rely upon chemotherapeutic agents like formalin, copper sulfate and potassium permanganate, which control fish disease by interrupting the life cycle of the parasite. But these chemicals now appear insufficient to control this parasitic infection (Madsen et al., 2000b) since the pathogens develop resistance towards the same through mutagenesis and plasmid mediated gene transfers (Toranzo et al., 1984) which may further activate teratogenic properties of those chemicals making them (Alderman, 1985; Wahli et al., 1993) toxic for the fish. It has been observed previously that long term use of antibiotics and other therapeutic drugs leads to bioaccumulation and residual formation in fishes (Goven et al., 1980; Klinger and Floyd, 2002). Therefore, many countries including India have also banned the use of antibiotics in aquaculture due to public health concerns and environmental hazards. Thus, there is an urgent need to systematically evaluate the plants used in traditional medicine as an alternative strategy for controlling trichodiniasis in aquaculture (Noor El Deen and Mohamed, 2009). After the herbal renaissance occurred all over the world, medicinal plants are staging a phenomenal comeback. Pharmaco

* Corresponding author at: Parasitology Laboratory, Department of Zoology, University of Kalyani, Kalyani 74123, West Bengal, India.
E-mail address: prabir0432@hotmail.com (P.K. Bandyopadhyay).

http://dx.doi.org/10.1016/j.aquaculture.2017.02.009
0044-8486/

botanical information from India estimates more than 6000 medicinal plant species forming about 40 % of the diversity that are used in its codified and folk health care traditions (Baker, 1970). Nowadays, the use of traditional medicinal plants has become a matter of great attention since they are the cheapest source of therapeutics with greater efficacy than chemotherapeutic agents and having much lower side effects at the same time (Balasubramanian et al., 2007, 2008; Caruana et al., 2012). Higher plants have always been the source of various medicinal compounds which play a dominant role in maintaining human health (Farombi, 2003; Abutbul et al., 2004). Over fifty percent of all modern clinical drugs are natural products in origin (Stuffness and Douros, 1982) playing a key role in drug development programs in the pharmaceutical industry (Baker et al., 1995; Dubber and Harder 2008; Harikrishnan et al., 2011a, b, c, d, 2012a).

Garlic is one of the few edible plants that not only got enormous scientific interest, but has also been recognized as an economically important medicinal plant by virtue of having a wide variety of secondary metabolites such as tannins, alkaloids, flavonoids, triterpinoids, saponins, fatty acids and essential oils which possess substances with antiprotozoan properties that can be used as potential alternative medicines (Harris et al., 2001; Mikail, 2010). It has also been suggested that ethanolic extracts from garlic used in allopathic medicine are potential sources of antiviral, antimicrobial and antitumor agents (Vlietinck et al., 1995). Allicin which is a major component of garlic is known to help in arresting pathogenesis (Jabar and Al-Mossawi, 2007).

The primary goal of this study is to evaluate the efficiency of ethanolic garlic extracts in curing trichodiniasis of goldfish under in vitro conditions and towards achieving that goal the acute toxicity of the ethanolic garlic extract was first assessed and then the accurate dose of the ethanolic garlic extract was determined which is effective in killing the trichodinid ciliates without hampering the health and growth of the goldfishes. In the present study three genus of trichodinid parasites namely *Trichodina*, *Tripartiella* and *Trichodinella* have been considered (Saha and Bandyopadhyay, 2016; Saha et al., 2016).

Although it is quite well known that garlic produces a diverse range of bioactive compounds which are responsible for health benefits, but its mode of action is less known and that can be better investigated if the active ingredients are characterized. In this connection, it must be mentioned that gas chromatography-mass spectrometry (GC-MS) can be a very useful technique for that purpose. Few previous studies have conducted GC-MS analysis of garlic bulb (Ameh et al., 2013; Mohammed, 2013; Syed and Bari, 2014) but most of them are qualitative in nature. But in this study thorough identification of the major bioactive compounds having anti protozoan activities has been done using gas chromatography-mass spectrometry (GC-MS).

2. Materials and methods

2.1. Collection of plants

The bulb of *Allium sativum* was collected from the cultivated land as well as the local market, dried for about 15–20 days and powdered mechanically with the help of the commercial stainless steel grinder. The dried garlic powder were then sieved with the help of fine sieved cloth and prepared for extraction through Soxhlet apparatus (Ghosh et al., 2016).

2.2. Preparation of plant extract

Firstly, 30 g of garlic powder was dissolved in ethanol, methanol and water separately at a ratio of 1:10 in the Soxhlet apparatus. The extracts obtained after a run of 6 h at 40–50 °C from each of the three different extracting solvents were filtered in a Buchner funnel with Whatman number1 filter paper. The filtrate in each case was then processed further to concentrate the extract using Rota evaporator at 50–55 °C, which was followed by drying in air or incubator. A stock solution of 1% was prepared by dissolving 1 g of crude extract in 100 ml of respective solvent, and preserved in 4 °C for further use in bonsai (Ghosh et al., 2016).

2.3. Qualitative screening

Phytochemical screening was carried out by subjecting 1 g each of the dried ethanolic, methanolic and aqueous extract to different test as described below.

2.3.1. Test for flavonoids: alkaline reagent test

Crude extract was mixed with a few drops of sodium hydroxide solution. An intense yellow color was formed. Yellow color turned to colorless by adding few drops of dilute acid, indicated the presence of flavonoids (Siddiqui and Ali, 1997).

2.3.2. Test for fat: saponification test

Few drops of 0.5N of alcoholic potassium hydroxide were added in small quantity of crude extract. A drop of phenolphthalein was added separately in the solution which was heated in a water bath for one hour. The formation of soap indicated the presence of fixed oils and fats (Debela, 2002).

2.3.3. Test for Tannins: gelatin test

1ml of 1% gelatin solution was added to 1ml clear filtrate of plant extracts dissolved in ethanol and treated with 2% NaCl solution. Precipitation disappears with the addition of excess gelatin, which confirmed the presence of tannin (Iyengar, 1995).

2.3.4. Test for alkaloids: Mayer's test

The alcoholic extract was evaporated to dryness and the residue was heated with 2% hydrochloric acid on a boiling water bath. The mixture was allowed to cool. It was filtered and subsequently treated with a few drops of Mayer's reagent (potassium mercuric iodide solution) (Siddiqui and Ali, 1997). The samples were then observed for the presence of turbidity or yellow precipitation (Evans, 2002).

2.3.5. Test for steroids and triterpinoids: Salkowski test

A few drops of conc. H_2SO_4 along with chloroform was mixed with crude extract, shaken well and allowed to stand for some time. Red color appeared in the lower layer indicated the presence of steroids and formation of a yellow colored layer indicated the presence of terpenoids (Debela, 2002)..

2.3.6. Test for phenolic compounds: ferric chloride test

Three drops of a mixture of 1 ml each of 1% ferric chloride ($FeCl_3$) and 1% potassium ferricyanide [$K_3Fe(CN)_6$] were added to 2 ml of the aqueous solution of the extracts. Formations of green or blue color indicate the presence of polyphenols (Debela, 2002)..

2.4. Experimental fish

70 healthy and 240 infected goldfish (Mucus taken from the surface of gill, body and fin was smeared on clean grease free slides with a drop of 0.25% NaCl solution and were determined microscopically for parasites. Heaviest infected fish were carried out for treatment experiment and non parasite or healthy fishes were carried out for acute toxicity test) *Carassius auratus*, weighting 4.38 ± 0.28 g, were collected from a local fish farm. All fish were kept in several aerated glass aquaria of 20 lit capacities at 26.6 ± 2.3 °C, pH 6.9 ± 0.1 with dissolved oxygen 6.0–7.8 mg/L and acclimatized under laboratory condition for seven days before use for the test. They were fed once at 1% body weight daily with commercially available fish pellet.