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Inhibitory effects of silver zeolite on *in vitro* growth of fish egg pathogen, *Saprolegnia* sp.

Seyed Ali Johari[1], Mohammad Reza Kalbassi[2], Il Je Yu[3]

[1]*Fisheries Department, Natural Resources Faculty, University of Kurdistan, Sanandaj, Iran*

[2]*Department of Aquaculture, Marine Science Faculty, Tarbiat Modares University, Mazandaran, Noor, Iran*

[3]*Institute of Nanoproduct Safety Research, Hoseo University, 165 Sechul-ri, Baebang-myun, Asan 336-795, Korea*

PEER REVIEW

Peer reviewer

Dr. Susan Gibson-Kueh, Lecturer (Aquatic Animal Health), School of Veterinary and Life Sciences, Murdoch University, South St., Murdoch, Perth, Western Australia 6150.
E-mail: S.Kueh@murdoch.edu.au

Comments

This is a well presented article on the *in vitro* antifungal properties of silver zeolite. The results obtained clearly demonstrated the significant *in vitro* inhibition of growth of a *Saprolegnia* sp. at 62 mg/L and marked growth inhibition at concentrations above 500 mg/L.
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ABSTRACT

Objective: To investigate the effects of powdered silver zeolite (SZ) on the *in vitro* growth of the fish pathogen *Saprolegnia* sp.
Methods: The antifungal activity of SZ was evaluated by determining the minimum inhibitory concentrations using two-fold serial dilutions of powdered SZ in a glucose yeast extract agar at 22 °C. The growth of *Saprolegnia* sp. on the SZ agar treatments was compared to that on SZ-free agar controls.
Results: The results showed that SZ had an inhibitory effect on the *in vitro* growth of the tested fungi. The minimum inhibitory concentration of SZ for *Saprolegnia* sp. was also calculated at 600 mg/L, which is equal to 0.06 percent.
Conclusions: SZ is a potential good candidate to replace teratogenic and toxic agents, such as malachite green in aquaculture systems.

KEYWORDS
Antifungal, *In vitro*, *Saprolegnia*, Silver zeolite, Aquaculture

1. Introduction

Reduction of fish diseases is undoubtedly very important for the future success of the aquaculture industry. Indeed, the largest cause of economic losses in aquaculture is diseased fish, and oomycete (water mould) infections rank second only to bacterial diseases in impact[1]. Oomycetes such as *Saprolegnia*les, including the *Saprolegnia*, *Achlya*, and *Aphanomyces* species, have been found responsible for fish infections in aquaculture, fish farms, and hobby fish tanks[2-8]. *Saprolegnia* is one of the most destructive oomycete pathogens for fish, being endemic to all freshwater habitats around the world and partly responsible for the decline of cultured and natural populations of

*Corresponding author: Seyed Ali Johari, Fisheries Department, Natural Resources Faculty, University of Kurdistan, Sanandaj, Iran.
Tel: +98-912-6268409
Fax: +98-871-6620550
E-mail: a.johari@uok.ac.ir

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salmonids, cyprinids, acipensers, and other freshwater fish[9].

Although malachite green is very effective for controlling fungal infections on the surface of fish skin and fish eggs[7], its potential teratogenicity[10] has limited its use to the treatment of non-food fish under an investigational new animal drug application held by the U.S. Fish and Wildlife Service, and its re-registration for the treatment of fungal infections in food fish is highly unlikely[3]. Currently, there are few registered aquatic fungicides other than malachite green. Formalin is not completely effective for controlling fungal infections in fish or fish eggs[3,7], in addition, there are also concerns about its effect on both the environment and the personnel who handles it[11]. Furthermore, the use of other fungicides, such as ozone, hydrogen peroxide, sodium chloride, iodophors, and copper is not widespread[3,12-14]. Therefore, more research is needed to identify new secure and effective aquatic fungicides. In this regard, the efficacy of many potential fungicides has been tested using *in vitro* screening methods[3,15,16], and it was found that *in vitro* tests correlated well with the *in vivo* conditions of surface infections of fish[2,17].

Recently, various inorganic antibacterial and antifungal materials containing silver have been developed and some are already in commercial use[18-23]. Among various antibacterial metals, silver is known to have a wide antibacterial spectrum and is relatively safe[24-26]. Silver, copper, zinc, and other antibacterial metals, when bound to inorganic carriers designed for slow release are far superior as inorganic disinfectants in terms of safety, duration of action and resistance to heat when compared with conventional organic ones[27]. For this reason, the development of inorganic bactericides and disinfectants composed of silver bound on various inorganic carriers for application in domestic and industrial fields is receiving extensive attention[28]. Inorganic carriers used include zeolite, apatite, phosphates, titanium oxides and glass. SiOx, which have a porous structure, can adsorb various ions and organic molecules easily and is expected to be one of the most promising carriers suitable for the development of high performance antibacterial and antifungal materials. Zeolite is a porous crystalline material of hydrated sodium aluminosilicate, which exhibits a strong affinity for silver ions (Ag^+) and can electrostatically take up to approximately 40% (w/w) Ag^+[29]. Zeolite containing silver ions [silver zeolite (SZ)] mixed into resins or synthetic polymers can provide antibacterial and antifungal properties[29,30]. Several studies have been conducted to utilize SZ in medicine and hygiene industry[31-34].

Accordingly, due to the lack of information on the antimicrobial effects of SZ on fish pathogens, the present study examined the inhibitory effect of SZ against *Saprolegnia in vitro*. Techniques for the indirect application of SZ in fish production systems are also presented.

2. Materials and methods

2.1. SZ

SZ type AJ10N (commercial name: Zeomic) was donated by Sinanen Zeomic Co. Ltd. (Nagoya, Japan). It contains 2.5% (w/w) of Ag^+ bound electrostatically to synthetic type-A zeolite (84%), and also contains 13.5% zinc. Average particle size of zeolite in this product is 2.5 μm.

2.2. Antifungal activity tests

A pure stock of fish *Saprolegnia* sp. previously cultured from rainbow trout eggs and characterized by the Department of Aquatic Animal Health, Veterinary Medicine Faculty, University of Tehran was used in this study[35]. The *Saprolegnia* sp. was cultured on a glucose yeast extract agar (GYA) and stored at 4 °C until use. The composition of the GYA included: 20 g/L agar, 10 g/L glucose, 2 g/L yeast extract, 2.04 g/L KH_2PO_4, and 0.596 g/L $Na_2HPO_4 \cdot 12H_2O$.

The antifungal effects of the SZ were evaluated by determining the minimum inhibitory concentrations (MICs) using the agar dilution method[17,36]. The agar dilution method have been known and recommended as a standard *in vitro* antifungal susceptibility tests by the National Committee for Clinical Laboratory Standards[37]. Briefly, agar plugs containing fungal hyphae of *Saprolegnia* (5 mm in diameter) removed from the edge of the pure stock were placed in the middle of depression spots on plates containing various concentrations of SZ and incubated at 22 °C. The maximal growth of *Saprolegnia* (colony diameter) was determined after 24, 48, and 72 h, respectively. To determine the inhibitory concentration range to be used, eight test concentrations of SZ 2 000, 1 000, 500, 250, 125, 62, 31, and 15 mg/L plus a negative control without SZ prepared on GYA plates in triplicate. The growth of *Saprolegnia* in the presence of the SZ was compared to that of the control.

As antifungal activity or fungal growth inhibitory effects were observed between 500-1 000 mg/L in the range-finding tests, more than six concentrations of SZ, including 500, 600, 700, 800, 900, and 1 000 mg/L, were selected and their inhibitory effects against *Saprolegnia* were checked after 24, 48, and 72 h in the same way as described above.

To evaluate the *Saprolegnia* growth in different concentrations of SZ, the area over which the *Saprolegnia* hyphae grew in the Petri dishes was determined and compared to that in the negative control as follows. In all cases, the mean and standard deviations were calculated

using Microsoft Office Excel 2007.

$$\textit{Saprolegnia}\ \text{growth index}(\%)=\frac{\text{Growth area of } \textit{Saprolegnia} \text{ on the plates in the SZ treatments}}{\text{Growth area of } \textit{Saprolegnia} \text{ on the plates in the control}}\times 100$$

3. Results

The SZ exhibited dose-dependent effects on the colony size of the *Saprolegnia*. The colonies grew well in the controls, where after 72 h the whole surface of the culture media (on 90 mm Petri dishes) was covered by *Saprolegnia* sp. hyphae. The results of the range-finding tests showed no difference in the *Saprolegnia* growth with concentrations of 15 and 31 mg/L SZ compared to the negative control (without SZ) (Figures 1 and 2). With SZ concentrations of 62 to 500 mg/L, the *Saprolegnia* growth showed a gradual decrease, and no growth was observed at an SZ concentration of 1 000 mg/L. Our results showed that growth inhibition of *Saprolegnia* sp. was significant at SZ concentration of 62 mg/L and little to no growth above 500 mg/L.

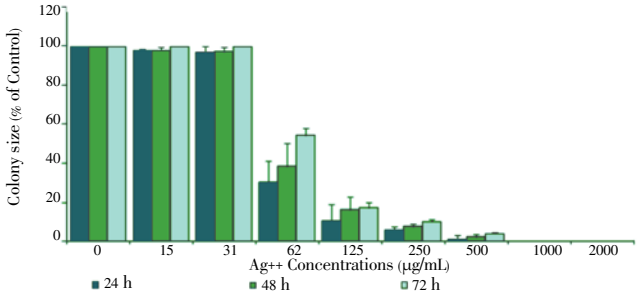

Figure 1. Growth of *Saprolegnia* in different concentrations of SZ, compared to control after 24, 48, and 72 h.

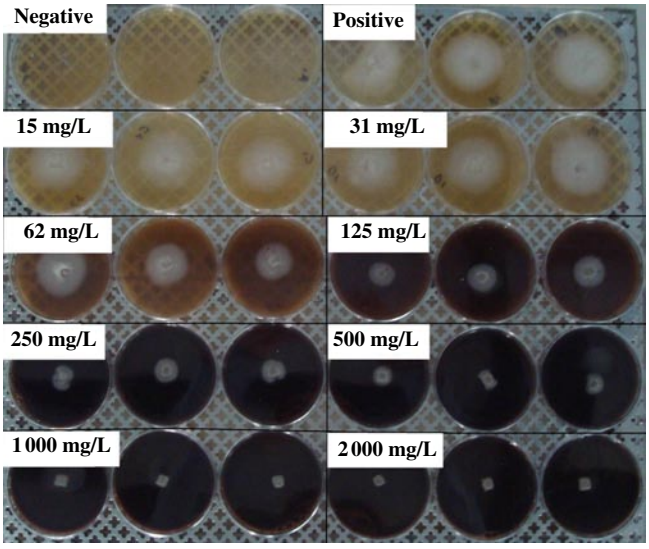

Figure 2. Growth of *Saprolegnia* on agar plates containing different concentrations of SZ (range finding test, after 48 h).

At 500 mg/L SZ, the *Saprolegnia* growth was less than 20% compared to the control, and at 1 000 mg/L, no growth was observed. Thus, the MIC of the SZ (at 22 °C) was calculated to be 600 mg/L for *Saprolegnia* sp., which was approximately equal to 0.06% SZ.

4. Discussion

Many studies have been done on the antibacterial and antifungal properties of various natural and inorganic substances such as tea extract, chitosan, copper and zinc, *etc*[38-40]. Silver and silver ions have been known to have powerful antibacterial and antifungal activity[41]. Silver is already commercially used to take advantage of its antibacterial properties[42]. SZ is widely used as an antimicrobial agent in home-electric appliances, toiletries, household goods, stationery, synthetic fibers and construction materials.

Unlike problems with antibiotics resistance, silver has been demonstrated to be a consistently effective antibacterial agent[43]. Several researchers have tried to explain the inhibitory effect of silver on bacteria, viruses, and fungi. It is generally believed that heavy metals react with proteins by combining the -SH groups of enzymes, which leads to inactivation of proteins. Feng *et al*.[44] investigated the inhibition mechanism of silver ions on microorganisms. Silver ions affect DNA molecules, causing the loss of replication abilities of DNA, and interact with thiol groups in protein thereby inactivating bacterial proteins.

In the present study, SZ was found to inhibit the *in vitro* growth of the water mould *Saprolegnia*, making SZ a potencial candidate for indirect use in the aquaculture industry; yet to confirm this supposition, further *in vivo* investigations are needed. Since SZ may have some toxic effects on fish, environment, and consumers, it is indisputable that it is not admissible to be used directly in aquaculture; but indirect methods can be applicable in aquaculture systems such as fish ponds, hatcheries, and aquariums industries. Based on the MIC results, mixing approximately 0.06% SZ into the structure of aquaculture equipment may minimize *Saprolegnia* growth. Now it is necessary to find the best applicable methods to use SZ in aquaculture systems, such as fish ponds, hatcheries, and aquariums. For instance, SZ can be incorporated in the filter media used in the water filtration systems of recirculation systems and hatcheries to control bacterial and fungal diseases transmitted and spread through water. Also SZ can be included in the polymeric structures of aquaculture systems, such as fiberglass or polyethylene troughs, trays, culture tanks, and other propagation and rearing instruments, as an antimicrobial and even antifouling agent that can reduce the use of anti-pathogen loads in the water environment.

In conclusion, further investigation of the antibacterial, antiviral, and antifungal activities of SZ against other

fish pathogens is needed. Furthermore, since the current experiment was carried out at 22 °C, the results correlate with the culture conditions of shrimp and warm water fish (such as cyprinids and acipensers). The current study needs to be repeated at lower temperatures (*i.e.* 10-12 °C) to understand the inhibitory effects of SZ and its potential use in systems for cold water fish species.

Conflict of interest statement

The authors declare that they have no competing interests.

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Comments

Background

Overgrowth of eggs by fungus of the family Saprolegniaceae is a common problem in fish hatcheries. Commonly used treatment often involves the use of toxic chemical compounds such as malachite green. As such, a compound with antifungal properties could be invaluable, especially if also found to be non-toxic.

Research frontiers

The present work presents the effectiveness of SZ as an antifungal agent against *Saprolegnia* sp. *in vitro*.

Related reports

As the authors of this article state under introduction, various inorganic antibacterial and antifungal materials containing silver have been developed and some are already in commercial use.

Innovations and breakthroughs

The potential value of an antimicrobial chemical compound such as SZ, if it can be proven to be relatively less toxic and has lower chances of development of drug resistance will be immeasurable.

Applications

The findings in this paper present SZ as a potential antifungal compound that can have wide applications. However, more research needs to be carried out to rule out the potential toxicity of this compound to live fish.

Peer review

This is a well presented article on the *in vitro* antifungal properties of SZ. The results obtained clearly demonstrated the significant *in vitro* inhibition of growth of a *Saprolegnia* sp. at 62 mg/L and marked growth inhibition at concentrations above 500 mg/L.

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