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Original Article

Study of fungicidal properties of colloidal silver nanoparticles (AgNPs) on trout egg pathogen, *Saprolegnia* sp.

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Abstract: Silver nanoparticles (AgNPs) are known to have bactericidal and fungicidal effects. Since, there is few information available on the interaction of colloidal nanosilver with fish pathogens. Hence, the current study investigated the effects of colloidal AgNPs on the *in vitro* growth of the fish pathogen *Saprolegnia* sp.. Before the experiments, various important properties of AgNPs were well-characterized. The antifungal activity of AgNPs was then evaluated by determining the minimum inhibitory concentrations (MICs) using two-fold serial dilutions of colloidal nanosilver in a glucose yeast extract agar at 22ºC. The growth of *Saprolegnia* sp. on the AgNPs agar treatments was compared to that of nanosilver-free agar as controls. The results showed that AgNPs have an inhibitory effect on the *in vitro* growth of the tested fungi. The MIC of AgNPs for *Saprolegnia* sp. was calculated at 1800 mg/L, which is equal to 0.18 percent. It seems that AgNPs could be a proper replacement for teratogenic and toxic agents, such as malachite green. In addition, the indirect use of AgNPs could be a useful method for providing new antifungal activity in aquaculture systems.

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Introduction

The reduction in fish diseases is undoubtedly very important for the future success of the aquaculture industry. Indeed, the largest cause of economic losses in aquaculture comes from diseased fish, and oomycete (water mould) infections are second only to bacterial diseases in their impact (Meyer, 1991). Oomycetes such as *Saprolegniales*, including the *Saprolegnia*, *Achlya*, and *Aphanomyces* species, have been found responsible for fish infections in aquaculture, fish farms, and hobby fish tanks (Baldauf et al., 2000; Bruno and Wood, 1999; Daugherty et al., 1998; Hussein and Hatai, 2002; Neish and Hughes, 1980; Okumuş, 2002; Willoughby and Pickering, 1977). *Saprolegnia* is one of the most destructive oomycete pathogens for fishes, being endemic to all freshwater habitats around the world and partly responsible for the decline of cultured and natural populations of salmonids, cyprinids, acipensers, and other freshwater fish (Van West, 2006).

Although malachite green is very effective for controlling fungal infections on the surface of fish and fish eggs (Okumuş, 2002), its suspected teratogenicity (Meyer and Jorgenson, 1984) has limited its use to the treatment of nonfood 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 (Bruno and Wood, 1999). 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 (Bruno and Wood,

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1999; Okumuş, 2002), plus there are also concerns about its effect on both the environment and the personnel who handle it (Fitzpatrick et al., 1995). Furthermore, the use of other fungicides, such as ozone, hydrogen peroxide, sodium chloride, iodophores, and copper is not widespread (Bruno and Wood, 1999; Forneris et al., 2003; Rach et al., 1998; Schreier et al., 1996). 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 (Bailey, 1984; Bailey and Jeffrey, 1989; Bruno and Wood, 1999), and it was found that *in vitro* tests correlated well with the *in vivo* conditions of surface infections of fish (Bailey, 1983a, b; Fereidouni et al., 2013).

Recently, various inorganic antibacterial and antifungal materials containing silver have been developed and some are already in commercial use (Hansel et al., 1998; Kawahara et al., 2000; Palenik & Setcos, 1996; Wang et al., 2007; Yamamoto et al., 1996; Johari et al., 2014a). Among various antibacterial metals, silver is known to have a wide antibacterial spectrum and be relatively safe (Cho et al., 2005; Mohan et al., 2007; Oya, 1996; TIC, 1998). One of the antimicrobial silver species that has been known for a long time, yet received little attention in aquaculture, is nanometer-sized silver particles (AgNPs) which exhibit both bactericidal and mycocidal effects (Cho et al., 2005; Mohan et al., 2007). Also recently antimicrobial efficacy of AgNPs have been studied against some fish pathogens (Vaseeharan et al., 2010; Antony et al., 2013; Mahanty et al., 2013; Dananjaya et al., 2014; Swain et al., 2014).

Since our previous study confirmed the prevention effect of silver zeolite (SZ) against *Saprolegnia* sp. (Johari et al., 2014a), in the present study we examined the inhibitory effect of AgNPs on the growth of the water mould *Saprolegnia* and found that nanosilver is effective against *Saprolegnia in vitro*. Suggestions are also given on techniques for the indirect application of AgNPs in fish production systems.

Materials and Methods

Silver nanoparticles and characterizations: Colloidal AgNPs, type L (commercial name: Nanocid), were donated by Nano Nasb Pars Co. (Tehran, Iran). The colloid product was synthesized using a process involving the photo-assisted reduction of Ag^+ to metallic nanoparticles, registered under United States Patent Application No: 20090013825 (Rahman Nia, 2009). According to information provided by the manufacturer, the product was a water-based colloid containing 4000 mg/L spherical AgNPs (average size 16.6 nm). The detailed specifications of this colloid have been analyzed and reported previously (Asghari et al., 2012; Johari et al., 2013). Also, prior to using the colloid product in the present study, TEM analyses of the undiluted AgNP suspension (4000 mg/L) were performed using an H-7100FA transmission electron microscope (Hitachi, Japan) with an acceleration voltage of 125kV. The diameters of 700 randomly selected particles were measured at a magnification of 100,000 using Axio Vision digital image processing software (Release 4.8.2.0, Carl Zeiss Micro Imaging GmbH, Germany).

Antifungal activity tests: A pure stock of fish *Saprolegnia* sp. previously purified from rainbow trout eggs and characterized by the Department of Aquatic Animal Health, Veterinary Medicine Faculty, University of Tehran (Shahbazian et al., 2010) 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 $Na_2HPO_4.12H_2O$.

The antifungal effects of the AgNPs were evaluated by determining the minimum inhibitory concentrations (MICs) using the agar concentration method (Bailey, 1983a, b). Briefly, agar plugs containing fungal hyphae of *Saprolegnia* (5 mm in diameter) were removed from the edge of the pure stock and placed in the middle of depression spots on plates containing various concentrations of AgNPs and incubated at 22ºC. The maximal growth of *Saprolegnia* (colony diameter) was determined after

24, 48, and 72 hours. To determine the inhibitory concentration range, twelve test concentrations of AgNPs, including 4000, 2000, 1000, 500, 250, 125, 62, 31, 15, 8, 4, and 2 mg/L plus a control were prepared on GYA plates in triplicate. The growth of *Saprolegnia* in the presence of the AgNPs was compared to that of the control.

According to the antifungal activity observed in the range-finding tests (inhibitory effects were observed between 1000–2000 mg/L), more six concentrations of AgNPs, including 1000, 1200, 1400, 1600, 1800, and 2000 mg/L, were selected and their inhibitory effects against *Saprolegnia* checked after 24, 48, and 72 hours in the same way as described above.

As a criterion for evaluating the *Saprolegnia* growth with the treatments, the area over which the *Saprolegnia* hyphae grew in the Petri dishes was calculated and compared to that in the positive control as follows. In all cases, the mean and standard deviations were calculated using Microsoft Office Excel 2007.

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

Results

Silver nanoparticles and characterizations: The AgNPs observed by TEM were spherical in shape, with a maximum diameter of 129 nm (Fig. 1): 65.14% of the particles had diameters between 1 and 13 nm (just 2.28% of the particles had diameters more than 100 nm) and the CMD (count median diameter) for the particles was 6.47 nm (Fig. 2). Also, the geometric mean diameter (GMD) and geometric standard deviation (GSD) of the AgNPs were 12.65 nm and 1.46, respectively.

Antifungal effects of silver nanoparticles: The silver nanoparticles exhibited dose-dependent effects on the colony size of the *Saprolegnia*. The colonies grew well in the controls, where after 72 hours the whole surface of the culture media (on 90 mm Petri dishes) was covered by *Saprolegnia* sp. hyphae. The

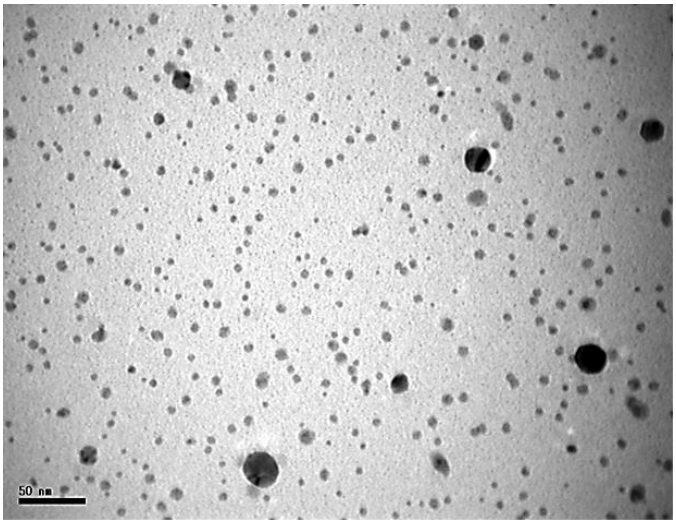

Figure 1. TEM micrograph of silver nanoparticles.

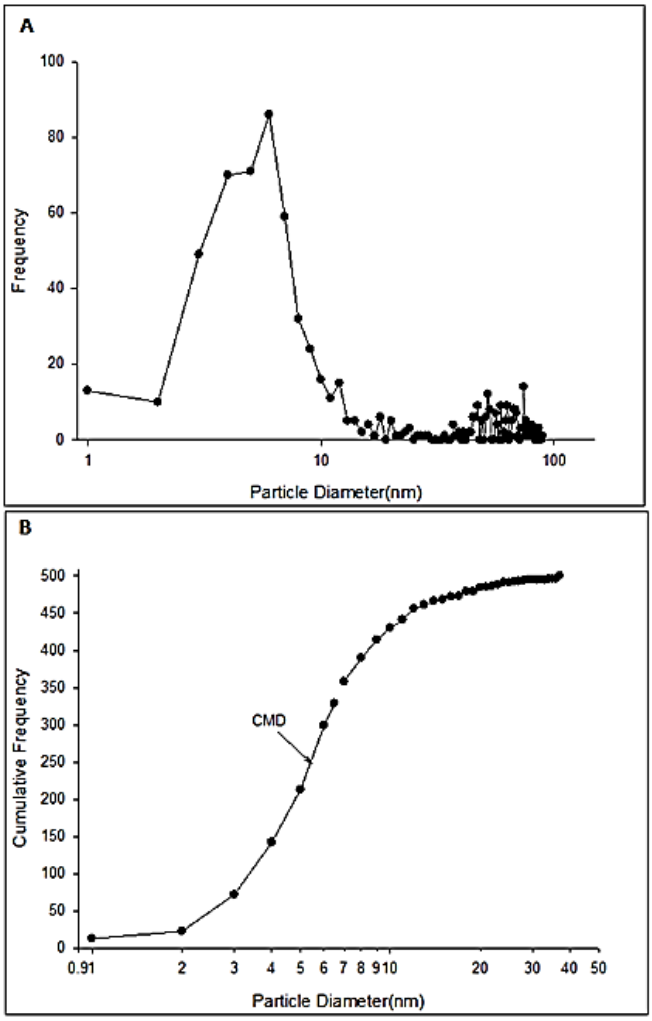

Figure 2. Size distribution of silver nanoparticles in undiluted suspension (4000 mg/L) based on transmission electron microscope data. A: Number Frequency and B: Cumulative Frequency (CMD: Cumulative median diameter).

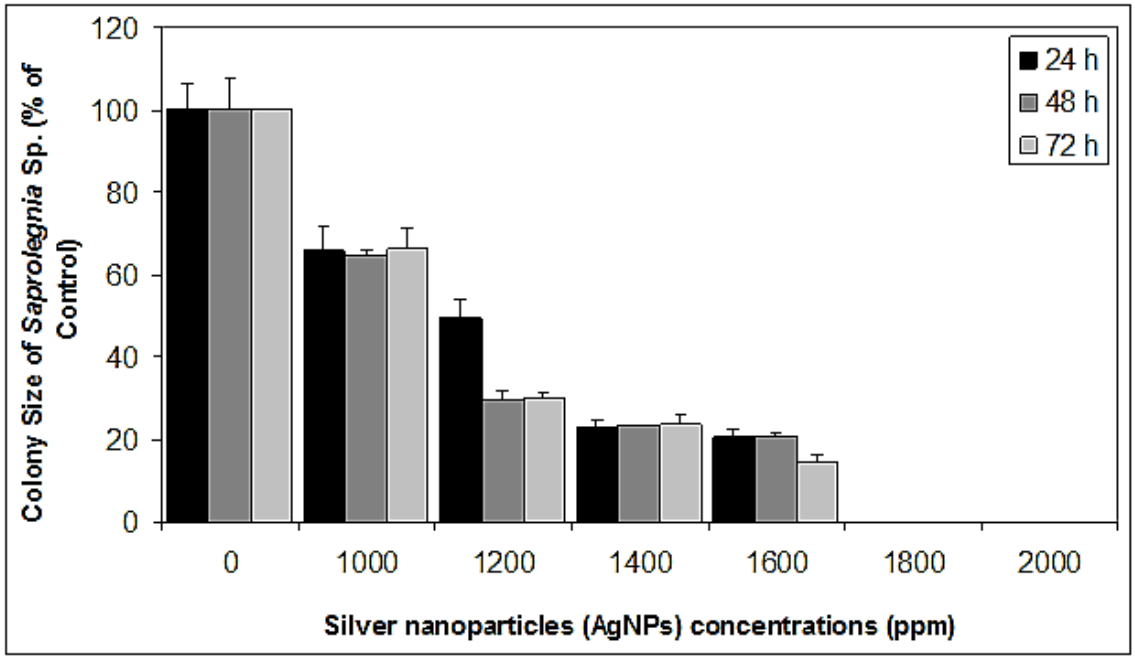

Figure 3. Growth of *Saprolegnia* in different concentrations of AgNPs, compared to control after 24, 48, and 72 hours (MIC determination test).

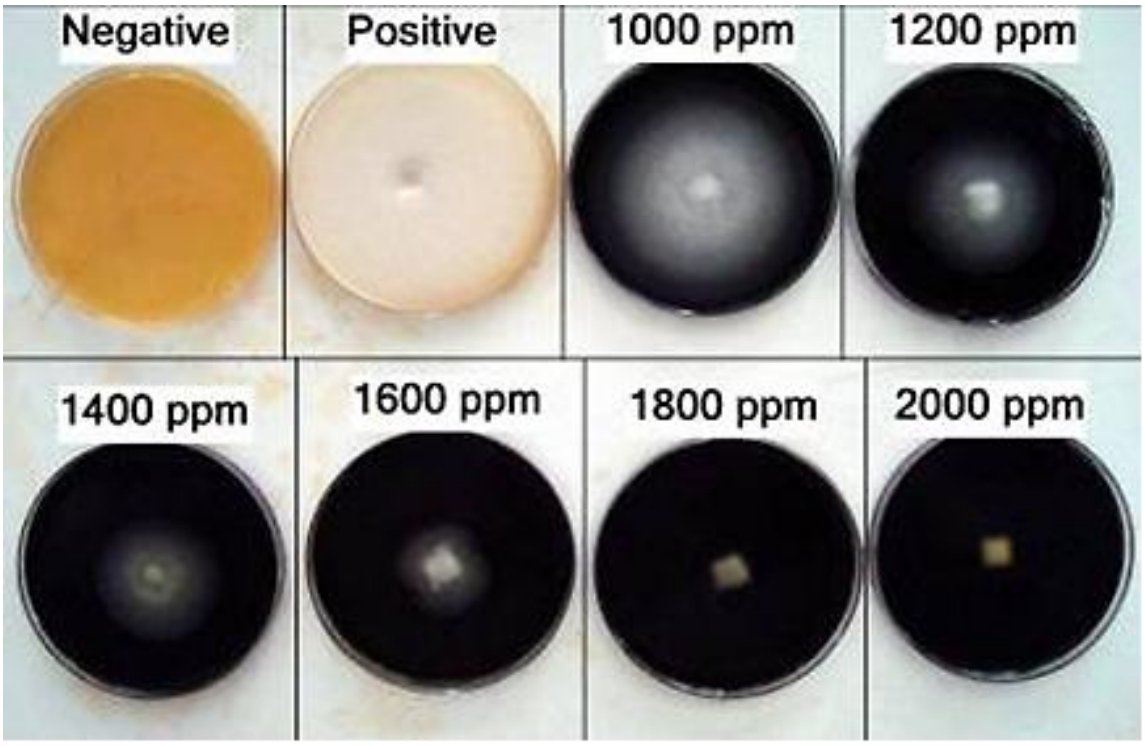

Figure 4. Growth of *Saprolegnia* on agar plates containing different concentrations of AgNPs (MIC determination test, after 72 hours).

results of the range-finding tests showed no difference in the *Saprolegnia* growth with concentrations of 2 and 4 mg/L AgNPs compared to the positive control (without AgNPs). Yet with AgNP concentrations of 8 to 1000 mg/L, the *Saprolegnia* growth gradually began to decrease, and no growth was observed with an AgNP concentration of 2000 mg/L. Therefore, it was concluded that growth of *Saprolegnia* sp. was limited within an AgNP concentration range of 1000 to 2000 mg/L.

The results of the MIC determination tests showed

that with 1200 and 1600 mg/L AgNPs, the *Saprolegnia* growth was less than 50 and 20%, respectively, compared to the control; and finally with 1800 mg/L, the *Saprolegnia* growth was zero (Figs. 3 and 4). Thus, the MIC of the AgNPs (at 22ºC) was determined to be 1800 mg/L for *Saprolegnia* sp., which was approximately equal to 0.18% silver nanoparticles.

Discussion

Silver particulates, and especially nanosilver in colloidal form, are known worldwide as a cure or inhibitor of bacterial, fungal, and viral diseases (Murr, 2009). Thus, silver is already commercially used to take advantage of its antibacterial properties (Kawashita et al., 2000). For example, silver nanoparticles are widely used in the production of antifungal and antibacterial ceramics, textiles, and paints. The mechanisms involved in the antimicrobial activity of AgNPs have previously been reported (Kim et al., 2007; Rai et al., 2009; Morones et al., 2005; Sanpui et al., 2008) and include: (1) changing and damaging the membrane structure of a microorganism, which increases its permeability and disrupts the transportation functions, resulting in cell death, (2) penetration of a microorganism and interaction with phosphorus and sulfur-containing compounds, such as DNA and proteins, (3) loss of the replication ability of the DNA, (4) inactivation of certain enzymes, (5) attacking the respiratory chain, (6) generating hydrogen peroxide and free radicals, and (7) the release of the silver ions from the nanoparticles, the antimicrobial activity of which is well known (Feng et al., 2000; Song et al., 2006; Yamanaka et al., 2005; Yoshihiro, 2002).

In the present study, AgNPs were found to inhibit the *in vitro* growth of the water mould *Saprolegnia*, making AgNPs a good candidates for indirect use in the aquaculture industry. It is important to note that the direct use of AgNPs in any form can pose several toxic effects on aquatic biota (Asghari et al., 2012; Johari et al., 2013; Johari, 2014; Johari et al., 2014b; Kalbassi et al., 2011; Salari Joo et al., 2012, 2013; Sharifian et al., 2013; Hosseini et al., 2014; Johari et al., 2015a; Tavana et al., 2014) and therefore the indirect use is the only way for peaceful use of this material in aquaculture industry. The MIC value for the AgNPs was high compared to that for other antifungal materials used for the direct treatment of eggs or larva (for example, malachite green), yet since AgNPs can be easily mixed or coated on other substances for indirect treatment, they may be a useful disinfectant against *Saprolegnia*. For instance, AgNPs can be included in the polymeric structures of aquaculture equipment, 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 ingredients in the water environment. Therefore, in terms of potential use, the incorporation of AgNPs into the surfaces and other objects in the aquaculture industry is conceivable. Based on the MIC results, mixing approximately 0.2% AgNPs into the structure of aquaculture equipment may completely inhibit *Saprolegnia* growth. Now it is necessary to find the best applicable methods to use AgNPs in aquaculture systems, such as fish ponds, hatcheries, and aquariums. Silver nanoparticles can be coated on the surface of different media (such as activated carbon, zeolite, and foams) and then used in the water filtration systems of recirculation systems and hatcheries to reduce bacterial and fungal diseases transmitted and spread through water. In this regard, Johari et al. (2015b) recently used a silver nanoparticles layer on the clinoptilolite and used this material in the water filtration system of salmonid's egg to prevent saprolegniasis.

In conclusion, further investigation of the antibacterial, antiviral, and antifungal activities of AgNPs against other fish pathogens is needed. Furthermore, since the current experiment was carried out at 22ºC, the results correlate with the culture conditions of shrimp and warm water fish (such as cyprinids and acipensers). Meanwhile, for cold water fish, such as salmonids, the current study needs to be repeated at lower temperatures (i.e. 10-

12ºC) to understand the inhibitory effects of AgNPs for such species.

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