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FULL LENGTH ARTICLE

Assessment of antifungal effects of copper nanoparticles on the growth of the fungus *Saprolegnia* sp. on white fish (*Rutilus frisii kutum*) eggs

Pegah Kalatehjari [a,*], Mahdi Yousefian [b], Mohammad Ali Khalilzadeh [c]

[a] *Department of Fishery, Qaemshahr Branch, Islamic Azad University, Qaemshahr, Iran*
[b] *Department of Biology, Payame Noor University, Sari, Iran*
[c] *Department of Chemistry, Qaemshahr Branch, Islamic Azad University, Qaemshahr, Iran*

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Abstract This study was conducted to evaluate the *in-vitro* effects of copper nanoparticles on the growth of the fungus *Saprolegnia* sp. isolated from white fish (*Rutilus frisii kutum*) eggs. The antifungal effects were measured by determining the minimum lethal concentration of copper nanoparticles on *Saprolegnia* sp. in yeast extract glucose chloramphenicol (YGC) agar at 25 °C. *Saprolegnia* grown in YGC agar without added copper nanoparticles served as negative controls. Our study showed that copper nanoparticles at a minimum concentration of 10 ppm have antifungal effects on *Saprolegnia* sp. The antifungal effects of copper nanoparticles are positively correlated to both concentration and time of exposure. This study showed that the antifungal properties of copper nanoparticles make it a good alternative to malachite green, which is carcinogenic.

Introduction

The restocking program of Caspian Sea white fish (*Rutilus frisii kutum*) in artificial program has been performed for more than two decades to preserve and recreate the resources of this valuable fish species as a vital wild fisheries one (Yousefian, 2004). Saprolegniasis is the most important fungal disease of freshwater fish and their eggs (Mokhayer, 1995). *Saprolegnia* sp. is often observed growing on the eggs of white fish (Yousefian, 2004). Despite known risks of malachite green as a teratogen and mutagen, it is still often used as a fungicide in some countries due to its high efficacy in controlling fungal infections (Sattari and Roustayi, 1999), but according to the U.S. food and drug administration it is not approved as an aquaculture veterinary drug in many countries including the United States, Canada and the European Union (2015). Considering the increasing growth of nanotechnology and its application in different sciences, it is required to apply this technology in fisheries and aquaculture research to take advantage of its positive effects in these fields. Nano-sized particles exhibit properties that are different from larger particles of

* Corresponding author.
E-mail address: pegah_kalatehjari@yahoo.com (P. Kalatehjari).
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the same substance, which are consequences of cutting the size of particles so as to increase their activity (Drexler, 1986). Copper nanoparticles are nanotechnology products with applications in various industries such as agriculture, livestock, household appliances, military and human medicine due to its anti-microbial properties (Borkow and Gabbay, 2009). Copper and its compounds are effective in removing a wide range of yeasts and fungi such as *Aspergillus carbonarius*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus oryzae*, *Candida albicans*, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* (Belli et al., 2006; Gershon et al., 1989; Kumbhar et al., 1991; Ohsumi et al., 1988; Vagabov et al., 2008; Zatcoff et al., 2008). Studies showed that antifungal properties of copper nanoparticles on *S. cerevisiae* (baker's yeast) are dependent on concentration (Cioffi et al., 2004).

To the knowledge of the authors, previous research on antifungal effects of nanomaterials has mostly been carried out in the field of medical science and on fungus *Candida* sp. (Lipovsky et al., 2011; Monteiro et al., 2011; Panacek et al., 2009; Paulo et al., 2010); However, there has been some research on the antifungal effects of silver zeolite, and silver nanoparticles in aquaculture. The minimal inhibitory concentration of silver zeolite was found to be 600 mg/L (or ppm) against *Saprolegnia* growth (Johari et al., 2014). Other beneficial effects of silver ions or nanoparticles include increased hatchability of fish eggs and larval survival (Soltani et al., 2010). The unsuitability of malachite green for use in aquaculture and emerging nanotechnology has led to increasing interests in nanoparticles as alternative fungicides. However, no research has been done so far on the usage of copper nanoparticles as a fungicide in aquaculture.

Materials and methods

Copper nanoparticle test concentrations used in this study

Colloidal copper nanoparticles at a concentration of 4000 ppm (Nanostructured Avizheh Company) were diluted with distilled water to the desired test concentrations of 4000, 2000, 1000, 500, 100, 50, and 10 ppm.

Source of Saprolegnia sp. used to evaluate antifungal activity of copper nanoparticles

Saprolegnia originally isolated from eggs of white fish (*Rutilus frisii kutum*) was propagated on yeast extract glucose chloramphenicol agar (YGC), in order to allow the use of identical fungal isolates for the different treatment groups. In summary, fungal colonies were placed on slides containing Lactophenol cotton blue (LPCB), covered with a coverslip, and examined under a microscope according to published methods (Dayal, 2001; Kitancharoen and Hatai, 1997; Roberts, 2001). The fungal isolates were identified using available identification keys (Dayal, 2001; Samson et al., 2000).

Fungal culture media preparation

According to Merck Millipore®'s instruction for yeast extract glucose chloramphenicol agar (YGC), 40 g of YGC-Agar medium powder was dissolved in 1 liter of distilled water, with heating and autoclaved at 121 °C for 15 min. Agar plates were stored at 4 °C until used. 5 ml of each copper nanoparticle test concentration was added to each plate, before *Saprolegnia* sp. was inoculated. In plate that served as a negative control, 5 ml of distilled water without copper nanoparticles was added.

Sampling and determining the rate of fungus growth

The diameter of fungal colonies was measured 12 hourly, for total duration of 72 h. The following equation was used for related calculations:

$$\text{Saprolegnia sp. growth index (\%)} = \frac{\text{Growth area of Saprolegnia sp. on the plate in the treatment group}}{\text{Growth area of Saprolegnia sp. on the plate in the control group}} \times 100$$

Data analysis method

The present study was conducted as a factorial experiment in a completely randomized design. Eight treatment and one control groups, consisting of three replications for each group were carried out. Results were obtained by taking the mean of the three replicates in each group. The data obtained were analyzed by using statistical analysis software (SAS) and Microsoft EXCEL. Comparison of mean results was performed using Duncan's multiple range test (DMRT).

Results

Fungal colony diameter was measured and recorded 12 hourly, for total duration of 72 h, which means it measured exactly at 12, 24, 36, 48, 60 and finally 72 h after *Saprolegnia* inoculation. Calculations were performed using equation 1. The effects of colloidal copper nanoparticle concentrations on the growth of fungi in comparison with the control group illustrate there is a significant decrease in relative colony size between 10 ppm Cu and negative control, but this was not so significant between 50 ppm and 200 ppm Cu, until the distinct no growth observed using 4000 ppm Cu (Fig. 1).

The relative colony size of *Saprolegnia parasitica* compared to the control group, at 12-hourly intervals for 72 h for all groups illustrates that fungal growth decreased over time compared to the control group (Fig. 2). It was also observed that the percentage of fungal growth was significantly different compared to the control group at all times ($P < 0.01$).

According to comparison of means, which was performed by using Duncan's multiple range test (DMRT), the relative colony size of treated groups compared to the control group at different Cu concentrations, over time illustrates that with increasing concentration and with passage of time, the percentage of fungal growth significantly decreased compared to the control group (Fig. 3). Treatment of 10 ppm showed no significant differences from control groups after 12 h ($P > 0.01$). However significant differences were observed in 10 ppm treatment groups after 12 h, and across all times for treatment groups at greater copper concentrations, when compared to the control group ($P < 0.01$).

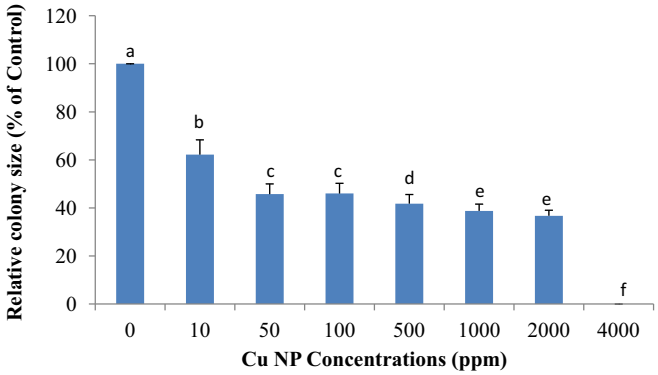

Figure 1 The effects of different concentrations of colloidal copper nanoparticles on relative colony size of *Saprolegnia* sp. compared to the negative control group using distilled water (0 ppm Cu). The means that are shown by different lower case letters indicate significant differences between treatments ($P < 0.01$).

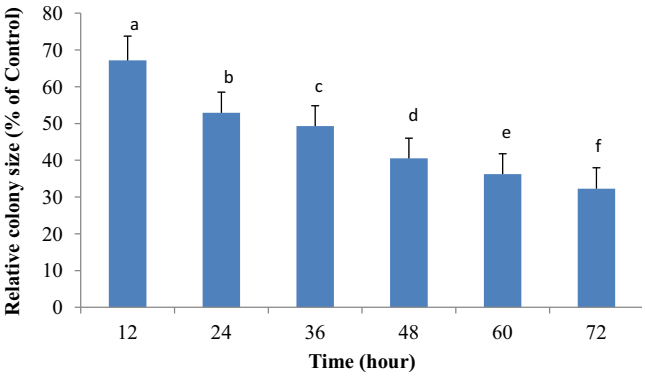

Figure 2 Relative colony size of *Saprolegnia* sp. compared to the control group, at 12-hourly intervals for 72 h for all treatment groups. The means that are shown by different lower case letters indicate significant differences between treatments ($P < 0.01$).

Discussion

Finding suitable substitute for malachite green is crucial for fish health, particularly White fish, which is ecologically precious species. Fungus belongs to *Saprolegnia* order or other orders may cause serious losses in fish hatcheries (Mokhayer, 1995). According to the results of this study, antifungal effects of copper nanoparticles went up by rising in the concentration, which was also observed in Cioffi et al. (2004) study; hence, in agreement with our results, Johari et al. (2014) illustrated that the best antifungal effects of nanoparticles occured in the highest concentrations. Consequently, this study as well as Soltani et al. (2010) study demonstrates the antifungal effects of nanoparticles in *Saprolegnia* sp. Also, antifungal activity of copper nanoparticles against selected crop and tomato pathogenic fungi in Kanhed et al. (2014) and Saharan et al. (2015) studies approves the efficiency of copper nanoparticles to apply as an antifungal agent, which is also concluded in the present study. Possible mechanisms of action of metallic copper, copper ions and colloidal copper nanoparticles are based on changes in the structure and function of the fungi cell; furthermore, these particles can affect DNA and disrupt its replication and transcription, which ultimately leads to the death of fungal microorganisms (Cioffi et al., 2004).

Based on the results obtained from this study, it is concluded that colloidal copper nanoparticles prevent the growth of the fungus *Saprolegnia* sp. in vitro. The minimum inhibitory concentration of the substance for the fungus *Saprolegnia*

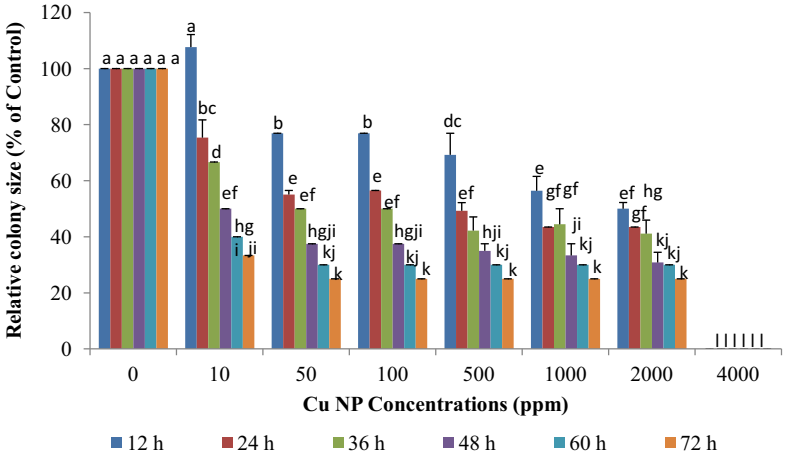

Figure 3 The interaction effects of Cu NP concentration and time on the percentage of *Saprolegnia* sp. fungal growth compared to the control groups. The means that are shown by different lower case letters indicate significant differences between treatments ($P < 0.01$).

in vitro is determined as 10 ppm. Consequently, the colloidal copper nanoparticles can be used as a treatment to prevent the growth of fungus *Saprolegnia* sp. Further research is required to find the most appropriate way of using this substance in aquaculture activities. Based on the results of this study, colloidal copper nanoparticles with concentration of 10 ppm can be used in the structure of aquaculture equipment such as fiberglass trough, rearing tanks or equipment for hatchery as an antimicrobial agent (bacteria and fungi). It can result in reducing consumption of the antifungal and antibacterial treatments in these environments. Further research is required in the field of anti-fungal and anti-bacterial colloidal copper nanoparticles against various pathogenic factors in the aquaculture industry. In addition, the exact mechanism of action of copper nanoparticles on aquatic pathogens has to be studied carefully.

In conclusion, Cu nanoparticles have antifungal effects on a *Saprolegnia* sp. isolated from white fish eggs. Antifungal effects are dependent on concentration used. Therefore the next steps would be to test what concentration of copper nanoparticles fish can tolerate.

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