Thesis for the Degree of Master of Fisheries Science

The efficacy of Virkon-S for the Control of *Saprolegniasis* in common carp, *Cyprinus carpio* L.

by

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KOICA-PKNU International Graduate Program Fisheries Science

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버콘-S의 잉어 물곰팡이병 치료 효과

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by

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Abstract

Saprolegnia parasitica is a severe fish pathogen that causes severe economic losses worldwide. Virkon-S is a well-known disinfectant that has known to have antimicrobial activities against bacteria, virus and fungi. In this study, we tested anti-fungal activity of Virkon-S against Saprolegnia parasitica, the major causal agent of saprolegniasis. In vitro assay showed that Virkon-S can inhibit the spore germination and the resulting mycelial growth at as low as 4 ppm. No cytotoxic effect on EPC cells was observed at as high as 100 ppm. Also, no acute toxicity on common carp was observed at 10 ppm during 96 h exposure. Virkon-S of 4 and 10 ppm showed complete treatment of Saprolegniasis artificially induced on common carp after 10 days' treatment. All this data indicates that

Virkon-S can be used for the control of saprolegniasis without harmful effect on fish although further researches on the effect on human and food supplies are necessary.

I. Introduction

Fungal infections are one of the main factors for mortality and economic losses among the ornamental and food fish farming industries (Fregeneda *et al*., 2007; Jalilpoor *et al*., 2005). The most common and economically important fungal disease of cultured fish is Saprolegniasis. Saprolegniasis usually occurs between October and March when water temperatures are below 15C^{o}, and the disease is also known as 'winter fungus'. Mortality usually increases as temperatures rise in early spring (Osman *et al*., 2008). *Saprolegnia* species can also infect dead fish eggs (Pottinger and Day, 1999). *Saprolegnia* infections are visible to the naked eye as white patches on the skin of the fish or as 'cotton wool' on fish eggs. From these eggs the fungus can spreads to live eggs via positive chemotaxis, meaning that some chemical signal from the live eggs causes the fungus to move towards them (Bruno and Wood, 1999).

This term 'Saprolegniasis' describes infection with fungi of the *Saprolegnia* complex belong to the oomycetes family Saprolegniomycetidae, which Most fish and animal pathogenic oomycetes belong to the Saprolegniomycetidae, which has two orders: Saprolegniales and Leptomitales. Within the Saprolegniales three main genera are recognized including *Saprolegnia, Achlya* and *Aphanomyces* and all are able to infect fish or shellfish (Daugherty *et al*. 1998). Although the species of *Saprolegnia* responsible for this disease has not yet been identified, *Saprolegnia parasitica*, *S. diclina* and *Achlya*

hoferi are the major etiological agents of saprolegniasis and *S. parasitica* is the most important among them (Van West, 2006). *S. parasitica* penetrate into epidermal tissues, usually colonizing the tail or head region and then proliferating to cover the entire body surface (Willoughby, 1994).

Traditionally, *S. parasitica* infections were effectively controlled with malachite green (Janos and Farkas, 1978; Srivastava and Srivastava, 1978; Alderman, 1985). However, the compound was banned worldwide in 2002, due to its undesirable effects on animal health (Van West, 2006, Brock and Bullis, 2001; Srivastava *et al*., 2004; Stammati *et al*., 2005). Since then, the search for new and effective substances against *Saprolognia* infections has been intensified. Although chemicals including formalin, hydrogen peroxide, and sodium chloride (Rach et al., 2005, Schreier et al., 1996, Barnes *et al.* 2003), copper sulfate (Straus et al., 2009), detergents such as bronopol (Pottinger and Day, 199), ozone (Forneris et al., 2003) have been shown to be somewhat effective, none of these alternatives were as effective as malachite green. Also, the use of these types of compounds has led to a number of problems, including the development of fungicide resistance and potentially harmful effects to human health (Stammati *et al*., 2005; Phillips *et al*., 2008), and still there is an urgent need to develop new alternatives that are effective in combating mycotic infections and also safe for the fish and environment (Khosravi et al. 2012).

Virkon-S was originally developed by Antec International and launched in 1986 to use for farming and livestock production, and is regarded as one of the most advanced farm

disinfectant. It was one of the first oxidative disinfectants to be used on the farm and continues to lead the way in livestock production and farm biosecurity, having been deployed successfully against 500 disease-causing pathogens including of viruses, bacteria and fungi including Foot and Mouth Disease (FMD), Avian Influenza, *Salmonella* and *Campylobacter* (Marchetti *et al*., 2006, Hernandez *et al*., 2000). For this wide range of antimicrobial activity and relative safety, Virkon™ S is selected by the United Nation's Food and Agriculture Organization and governments worldwide to secure biosecurity and strengthen Emergency Disease Control (EDC) contingency planning (http://www.virkon.com/en/products-applications/disinfectants/virkon-s/). In Korea, Virkon S has been approved as quasi-drugs for animal that can be used as disinfectants for aquaculture facilities in 2016.

Despite of wide spectrum of antimicrobial activity of Virkon-S, there has been no report about antifungal activity of Virkon-S against Saprolegnia. In this study we tested antifungal activity of Virkon-S against *Saprolegnia parasitica in vitro* and *in vivo* to find the possibility of using this material for the control of saprolegniasis in future.

II. Materials and methods

2.1. *Saprolegnia parasitica* cultures

Saprolegnia parasitica was purchased from the Korean Collection for Type Cultures (KCTC 46452) and cultured on potato dextrose agar (PDA) at 25°C. This strain was isolated from a farmed rainbow trout, Wonju, Gangwondo, Korea in 2016 before deposition.

2. 2 Fish and rearing conditions

One hundred fingerlings of common carps, *Cyprinus carpio,* with average size and weight of 17.6 ±3 g and 11.5±1 cm, respectively were obtained from Namsangju Aquaculture Farm, located in Sangju-si, Gyeongsangbuk-do, Korea. Each fish was examined for any infection and then acclimated in 450 L rearing tanks at 22 ±2°C for 10 days. During the acclimation, fish were fed to apparent satiation twice daily with a proper diet according to (Ellsaesser *et al*., 1986).

2.3. Virkon-S

Virkon-S was purchased from Korean Bayer Co. and the contents in 1kg is as follow; Triple salt 500 g, Hexametaphosphate 181g, Sodium dodecyl benzene sulphonic acid 150 g, malic acid 100 g, sulphamic acid 50 g, sodium chloride 15 g. A 10% stock was prepared with distilled water and further dilution was made when it is necessary.

2.4. Determination of minimum inhibitory concentration (MIC)

Minimum concentration of Virkon-S that inhibits the growth of *S. parasitica* was determined by modified methods of Zahran and Noga (2010). *S. parasitica* was cultured on PDA at 25C for 12 days to induce zoospores. About 10ml of distilled water was added to each 87mm diameter petri dish and filtered through 8 layers of sterile cheese cloth and the final concentrate of the zoospore were adjusted to approximately 1×10^6 zoospores/ml. One milliliter of zoospore suspensions was added the wells of to a flat-bottomed 6-well polystyrene plate and 1ml of diluted Virkon-S which are twice of the final concentration were added the wells to make the final concentration of 10, 20, 100, 200, 1000ppm. One milliliter of distilled water was added to negative control and experiment was conducted in triplicates. One hundred microliter of potato dextrose broth (PDB) was added to each

well and the plate was incubated at 25°C, and all wells were scored for the presence or absence of water mold growth 48 h after the start of the experiment.

2.5. Mycelia growth inhibition on PDA plates

Inhibition of S. parasitica mycelial growth was tested on PDA plates containing different concentration of Virkon-S by methods described by Hu *et al*. (2013). 2X PDA was sterilized and cooled down to about 65°C and mixed with the same volume of Virkon-S to give final concentration of 2, 4, 10, 20, 100 ppm. PDA with 2X concentration was mixed with the same volume of sterile water in control plates. Aliquots of 10 ml of the mixture were poured onto petri dish L) of treated medium were poured into each 87mm diameter. A S*aprolegnia*-colonized PDA block of about 5x5 mm was placed in the center of the prepared plates. The plates were incubated at 25°C for 72 h and the diameter of the mycelia was measured. The percentage of fungal inhibition was calculated based on percent inhibition of radial growth (PIRG) as described by Dananjaya et al. (2017) which is as follows: PIRG (%) = [(R1 − R2)]/R1 × 100%, where, R1 = radial growth in control, and R2 =radial growth in treatment.

2.6. Inhibition of spore germination

Inhibitory effects of Virkon-S on spore germination were tested in two methods. First, the minimum oomyceticidal concentration (MOC) defined as the lowest concentration of the chemicals that prevented visible growth or germination of spores was determined as described by Hu *et al*. (2013). Spores of *S. parasitica* were produced and harvested as described above and the concentration of the spores was determined using a hemocytometer and adjusted to approximately 1×10^6 spores/ml. Petri dish of 87mm in diameter containing 20ml of PDA with 0, 2, 4, 10, 20, 100 ppm Virkon-S and were prepared as above. Ten microliter of spore suspension were spotted in the center of each plate and then incubated at 25°C. After 72 hours' incubation, the growth of mycelia was observed with naked eyes

Inhibition of spore germination was also determined by percent spore germination as described by Kiraly *et al*. (1974). PDA containing 0, 2, 4, 10, 20, 100 ppm Virkon-S and were prepared but the amount of total PDA was only 10 ml for each plate. This condition reduced the mycelial growth and the plate was transparent enough to observe spore germination under a light microscope. Three spots of 10 µl spore suspension were placed on each plate of 0, 2, 4, 10, 20, 100 ppm Virkon-S and then incubated at 25°C for 72 h. The Percent spore germination was determined as follows:

No. of spores germinated

$$\text{Percent spore germination} = \frac{\text{Total no. of spores examined}}{\text{Total no. of spores examined}} \times 100$$

2.7. Cytotoxicity of Virkon-S on Epithelioma Papulosum Cyprini (EPC) cell

Cytotoxic effect of Virkon-S was evaluated using the Ez-Cytox Cell Viability Assay Kit (Dogen-Bio Co., Ltd., Seoul, Korea) and EPC cells following the procedures of the manufacturer as described by Park et al. (2017). EPC cells (1×10^5) were grown in L-15 medium supplemented with 10% FBS and antibiotics in 96-well plates overnight. The cells were treated by replacing the medium with medium (100μL) containing 10, 100, 500, 1000, 5000 and 10000 ppm of the Virkon-S. Non-treated cells were used as the negative control. After 24 h of incubation, 110 μL of medium containing 10 μL of water-soluble tetrazolium solution were added to each well, and the plates were incubated for a further 4 h. Absorbance at 460 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Silicon Valley, CA, USA), and relative cell viability was calculated using cells treated with medium only as a control.

2.8. Acute toxicity against common carp

Water-only toxicity tests were carried out with cultured fingerlings of common carps, *Cyprinus carpio* using 5 concentrations of Virkon-S, 2, 4, 10, 20 and 100 ppm, and three fishes in each concentration. Healthy and disease-free fish that were not previously exposed to any pollution agents or toxicants were selected based on their activity and external appearance. Fishes were acclimated 7 days at 18ºC under constant light and feeding of commercial feed. Three fishes were placed in a 3-liter glass flask containing different concentration of Virkon-S and kept for 96 h aerated water without water change or feeding. However, any dead fish was removed from the flask to possible deterioration of the water quality (Hedayati *et al.,* 2010). Mortality was recorded after 24, 48, 72, 96 h and LC_{50} values and its confidence levels (95%) were calculated. Percentages of fish mortality were calculated for each concentration at 24, 48, 72, 96 h of exposure. LC_{50} values were calculated from the data obtained in acute toxicity bioassays, by Finney's (Hedayati *et al*., 2010). After 96 h exposure, all survivors were transferred to 50-L aerated tank equipped with aeration (Yao *et al*., 2010) and observed for 40 days to see any aftereffect of the exposure.

2.9. Inhibition of fungal infection by Virkon-S in tank

Experimental infection of fish

Five groups of 20 fish were used for artificial infection of *S. parasitica* and treatment with Virkon-S. Three groups were artificially infected and treated with 2, 4, 10 ppm Virkon-S. Fishes in the positive control group were infected with *S. parasitica* but not treated with Virkon-S. Fishes in the negative control group were neither infected with *S. parasitica* nor treated with Virkon-S. Fishes in treatment groups were kept in a 150L glass fiber tank that is separated into three sections by metal screen with 6 or 7 fishes in each section. Fishes in the positive and negative control were kept in separated 50L tanks with 6 or 7 fishes per tank. The water temperature was fixed at 17±1°C by room air-condition. All fishes were observed for behavioral and clinical signs of infection.

For artificial infection with S. parasitica, fishes were descaled and wounded in 3 positions in one sides of body, top of head, center of body and the tail region using a sharp scalpel to make the wounded area approximately 1cm^2. Zoospores of *S. parasitica* were prepared as above and added to three treatment groups and the positive control group to the final concentration of 1×10^5 zoospores/mL (Willoughby, 1994; Hatai *et al*., 1994).

Confirmation of infection by *S. parasitica*

Ten days after addition of the zoospores, white cotton wool like white growth on the surface of wounded area was taken and observed with a light microscope (Hussein *et al*., 2001). Also, it was cultured on PDA and incubated at 25°C for 3 days. The morphological and microscopic characteristics of the culture were compared with the characteristics of S. *parasitica* recorded on references. (Hatai *et al*., 1990; Willoughby *et al*., 1984).

Treatment of *Saprolegniasis* with Virkon-S

To assess the effect of Virkon-S on controlling and treatment of artificially induced *Saprolegniasis* on common carps Virkon-S solution was added to the tanks at the final concentration of 2, 4 and 10 ppm. The water in the tank was replaced with same concentration after 5 days. The cumulative mortality was recorded for 10 days and fishes were checked and diagnosed by clinical signs and lesion healing on 10 days after treatment.

III. Results

3.1. Determination of minimum inhibitory concentration (MIC)

The minimum concentration of Virkon-S that necessary to inhibit the zoospore germination or growth of *S. parasitica* in distilled water was determined after 72 h of incubation at 25C. There was no germination or growth of *S. parasitica* in the concentration of 10, 20, 100, 200 and 1000 ppm. Instead of zoospore germination or mycelial growth bulging of the spores were observed in this concentration (Fig. 1A-D). Spore germination and mycelial growth was observed at 5 ppm (Fig. 1E) but mycelial growth was less than the mycelial growth in control (Fig. 1F). Therefore, the MIC of Virkon-S against *S. parasitica* growth was 10 ppm.

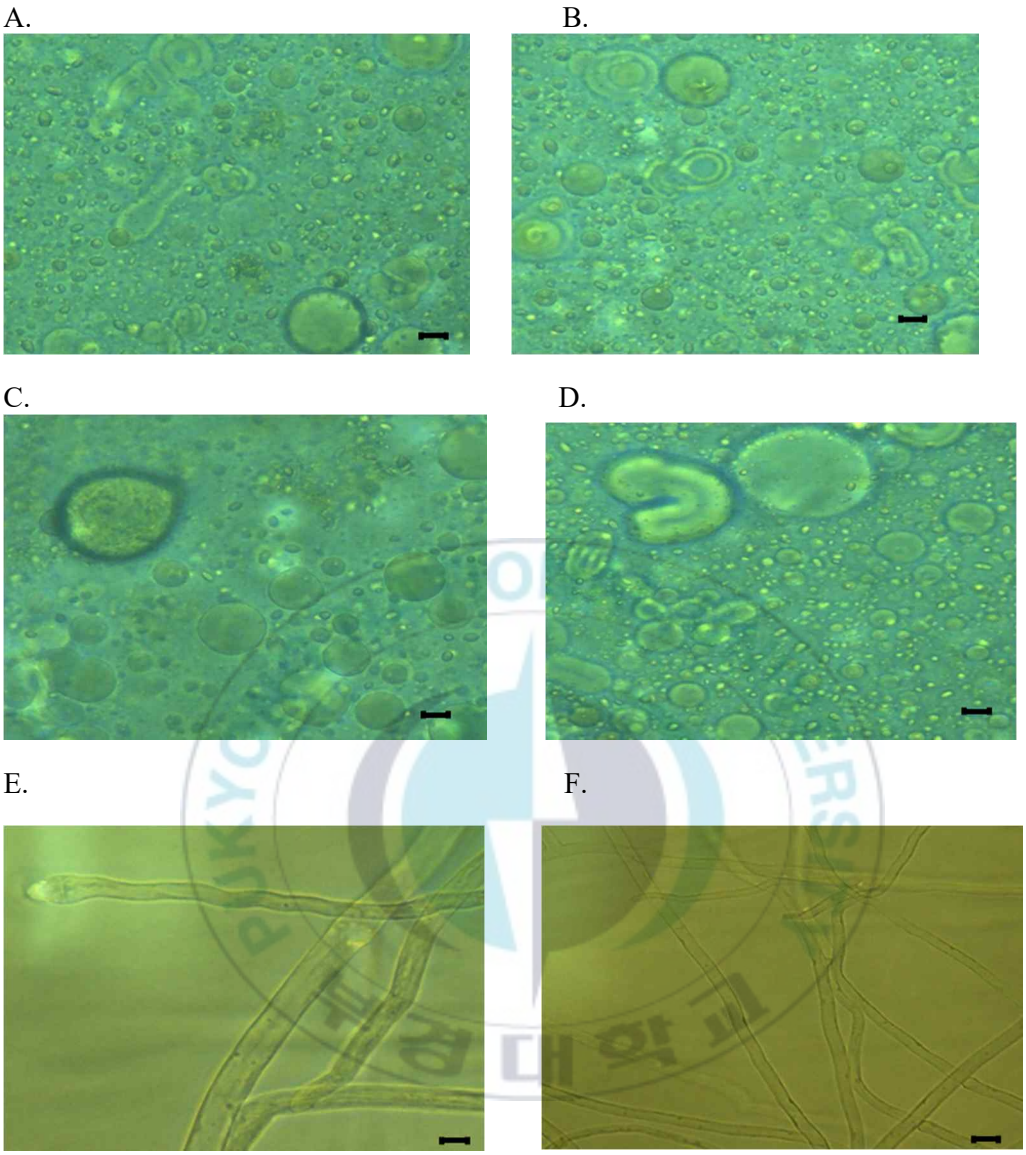


Figure 1. Spore germination and mycelial growth of S. parasitica in the presence of Virkon-S. A; 1,000 ppm, B; 200 ppm, C; 100 ppm, D; 20 ppm, E; 10 ppm, F; control. The bars in the picture are 1um.

3.2. Mycelia growth inhibition test

The growth of *S. parasitica* on PDA containing 100, 20, 10, 4, and 2 ppm Virkon-S was observed after inoculation at 25 °C for 72 h. No growth of mycelia was observed in the plate containing 100, 20, 10 ppm Virkon-S (Fig. 2A-C). There was slight growth of *S. parasitica* on PDA containing 4 ppm Virkon-S (Fig. 2D). The growth inhibition rate (IR) on the plate containing 2 ppm was 87.5% when the IR was calculated as %IR = 100 (X-Y) / (X-Z), where X, the mycelia growth in control; Y, the mycelia growth in sample; and Z, the average diameter of the inoculum.

Figure 2. Inhibition of *S. parasitica* mycelia growth on PDA containing different concentration of Virkon-S. A; 100 ppm, B; 20 ppm, C; 10 ppm, D; 4 ppm, E; 2 ppm. No Virkon-S was added in the control plate.

3.3. Inhibition of spore germination on PDA plates

Inhibition of zoospores germination of *S. parasitica* by Virkon-S was tested on PDA plates. Ten microliter of spore suspension containing 1×10^6 zoospores/ml was placed on the center of Petri dishes containing 0, 2, 4, 10, 20, 100 ppm Virkon-S. Spore germination and the resulting mycelial growth was observed after 72 h after incubation at 25C. There was no sign of mycelial growth on plates containing 4, 10, 20, 100 ppm Virkon-S (Fig. 3A-C). Mycelial growth on a plate containing 2 ppm was 53.8% of the control plate (35mm vs 65mm, respectively) (Fig. 3D and E). Therefore, the minimum oomyceticidal concentration (MOC) was defined 4 ppm on PDA plates.

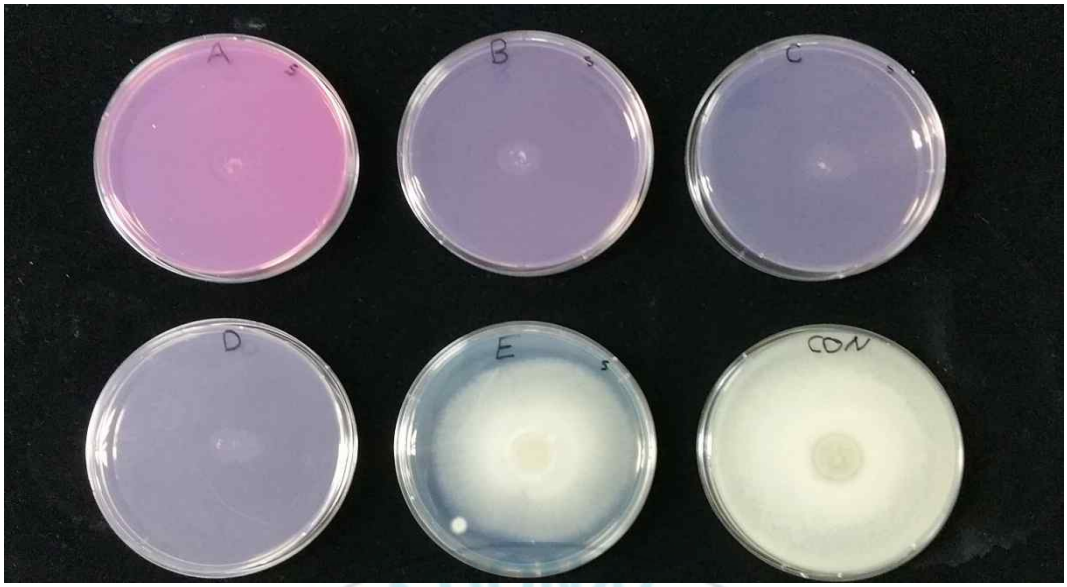
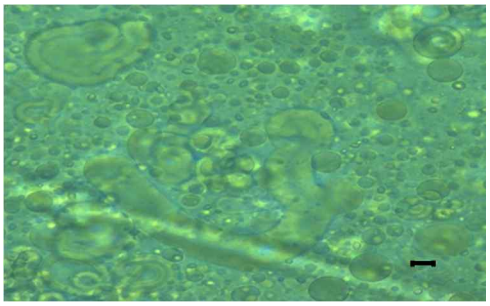


Figure 3. Inhibition of zoospore germination and the resulting mycelial growth on PDA plates by different concentration of Virkon-S. The pictures are 72 h after incubation at 25°C. A; 100 ppm, B; 20 ppm, C; 10 ppm, D; 4 ppm, E; 2 ppm. No Virkon-S was added in the control plate.

3.4. Enumeration of the inhibition of spore germination

In order to enumerate the inhibition of spore germination, PDA plates containing 0, 2, 4, 10, 20, 100 ppm Virkon-S were prepared by adding only 10ml of PDA mixture on an 87mm plates. This amount of medium was transparent to observe the spores on the surface by using alight microscope. Ten microliter of spore suspension containing 1×10^6 zoospores/ml was placed on three spots per plate and incubated 72 h after incubation at 25C before observation. There was no sign of spore germination on plates containing 4, 10, 20, 100 ppm Virkon-S (Fig. 4A). Instead, bulging of spores observed in distilled water containing Virkon-S was also observed on PDA plates. Small number of zoospores on PDA containing 2 ppm germinated with the germination rate of 39.2% (22 out of 56) (Fig. 4B). In contrast, most of the spores on control plates germinated with the germination rate of 96.2% (51 out of 53 spores) (Fig. 4C). Therefore, the inhibition rate of spore germination was calculated as 59% at 2 ppm concentration (96.2-39.2)/96.2x100.

A.



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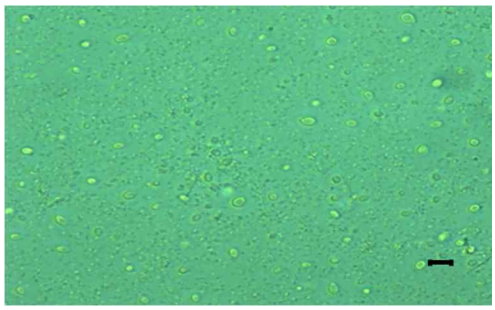

C.

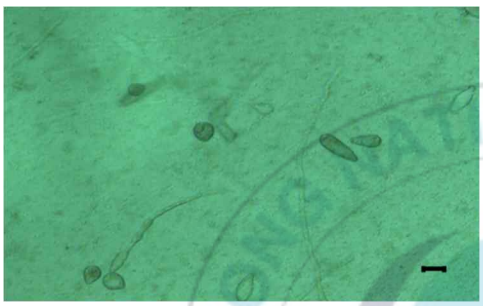

Figure 4. Inhibition of zoospore germination and on PDA plates by different concentration of Virkon-S. The pictures are 72 h after incubation at 25°C. A; 4 ppm, B; 2 ppm, C; control plate with no Virkon-S. Bars in the pictures represent 1um.

3.5. Cytotoxicity of Virkon-S on cultured EPC cells

The cytotoxic effect of Virkon-S on EPC cells, one of most widely used fish cell lines, was measured with Ez-Cytox Cell Viability Assay Kit (Dogen-Bio Co., Ltd., Seoul, Korea) and the result is shown on Fig. 5. There was no effect of Virkon-S on the cell viability at 10 and 100 ppm. There was slight decrease (97%) at 500 ppm. However, the cell viability dropped over 1000 ppm. In the mycelial growth inhibition and spore germination inhibition assay, 4 ppm was enough to inhibit zoospore germination and the resulting mycelial growth, which was much lower than 100 ppm that still did not show any toxicity to EPC cells. Therefore, 10 ppm and lower concentrations were used in further experiments.

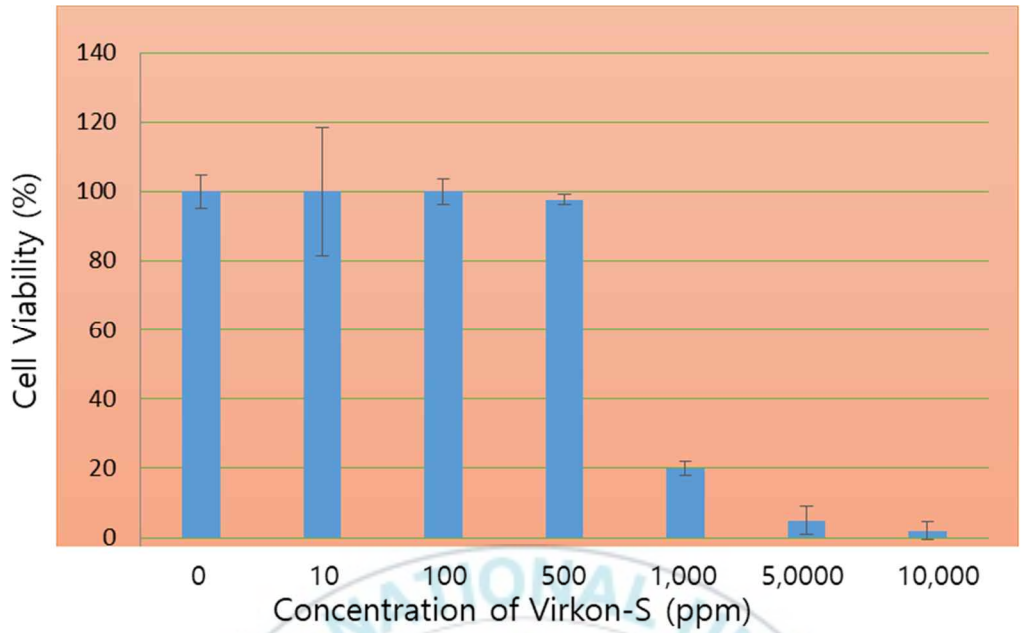

Figure 5. Cytotoxic effect of Virkon-S on cultured *Epithelioma Papulosum Cyprini (EPC)* cells. The results are the average from 8 replications and the standard deviations are indicated.

3.6. Acute toxicity test of Virkon-S on common carp

Acute toxicity of Virkon-S on common carp was investigated by placing the fish in glass flask containing five different concentrations of Virkon-S for up to 96 h. As shown on Table 1, all fishes in 100 ppm flask died within 24 h. Also, 75 and 100% cumulative was observed in 20 ppm flask. However, there no mortality was observed in all other flask containing 10, 4, and 2 ppm Virkon-S. Furthermore, the survivors from the acute toxicity test did not show any after effect when they were kept in culture tank for 40 days. So, it was concluded that 10 ppm which is enough for the inhibition of zoospore germination and mycelial growth but did not show any cytotoxic effect on EPC cell can be the maximum acceptable toxicant concentration (MATC) for Virkon-S. Fishes in the test flasks are shown in Fig. 6.

Table 1. Cumulative mortality of common carp during acute exposure to Virkon-S

Concentration (ppm)	Cumulative Mortality (%)			
	24 h	**48 h**	**72 h**	**96 h**
100	100	-	-	-
20	75	100	-	-
10	0	0	0	0
4	0	0	0	0
2	0	0	0	0

Figure 6. Common carp in the progress of acute toxicity test. Picture was taken after 48 h exposure and the leveling represent the dilution factor that make the final concentration of 100, 20, 10, 4, 2 ppm respectively.

3.7. Induction of artificial infection and treatment with Virkon-S

Seven days after addition of *S. parasitica* zoospores to the tanks containing artificially wounded common carp, the typical sign of saprolegniasis including cotton shape growth of fungi and ulceration of wound appeared (Fig. 7). The cotton wool was taken from the wound and observed with a microscope to confirm that it is the inoculated *S. parasitica*. Also, it was placed on a PDA plate to induce mycelial growth. Both of the cotton wool from the infected fish and mycelia grown in PDA showed the same morphological characteristics of *S. parasitica*, which indicated that the disease was induced by the zoospores of *S.parasitica* that had been added to the tanks (data not shown). The diseased fish were treated with Virkon-S at the final concentration of 2, 4, 10 ppm and the results are shown on Table 2. During 10 days' treatment, no mortality was observed among fishes being treated with 4 and 10 ppm. However, cumulative mortality in the 2 ppm tank reached 50%. Furthermore, fishes in the 4 and 10 ppm showed clear recovery from the disease. As shown on Fig. 8, all of the cotton shape saprolegniasi*s* of the disease disappeared on the fish's body. Furthermore, new scales appeared and covered the wounded area, which indicated complete recovery from the disease.

Figure 7. Clinical signs of *S. parasitica* in on fish body that had been artificially wounded and exposed to the fungal zoospore. The picture is typical sign of infection after 10 days' inoculation with cotton wool shape growth of the fungi on wounded area.

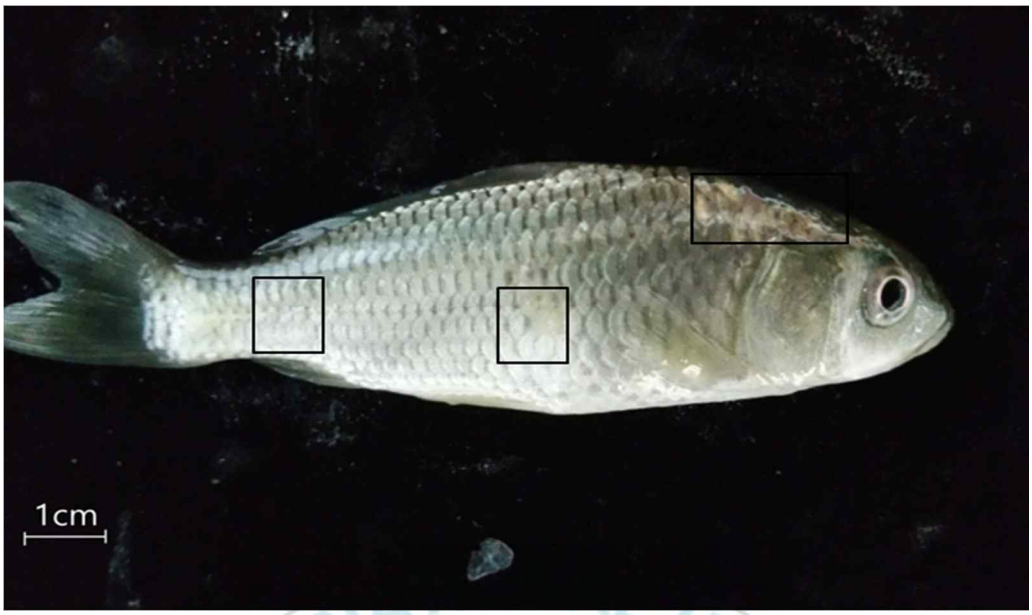

Figure 8. Common carp recovered from saprolegniasis by treatment with Virkon-S. This fish was treated with 10 ppm Virkon-S for 10 days after artificial inoculation with S. parasitica. The rectangles indicate wounded area for artificial infection and show complete recovery from the disease.

Table 2. Healing of artificially induced saprolegniasis by Virkon-S treatment

Treated Concentration (ppm)	% of healing after treatment	Cumulative mortality (%)
10	100	0
4	100	0
2	50	0
Positive control*	0	90
Negative control**	-	0

*Positive control group was artificially infected with S. parasitica zoospores but was not treated with Virkon-S

**There was neither artificial infection nor treatment Virkon-S.

IV. Discussion

Since the ban of using malachite green for the treatment of saprolegniasis because of its possible genotoxic carcinogenesis and residual toxicity, this disease has resulted in severe economic losses in fresh water fish farming (Van West, 2006). Although many alternatives have been tried, no chemicals presently available that give sufficient protection against the disease. In addition, sanitary problems, environmental restrictions, and high cost have also limited the use of these synthetic antimicrobials (Yao et al., 2017). The only U.S. Food and Drug Administration (FDA)-approved compounds for fungus control are 37% formalin and 35% hydrogen peroxide, but the efficiency is lower than malachite green (Straus et al., 2016).

Virkon-S is one of the well-known disinfectant s that has been proven to be effective against bacteria, viruses and fungi (Gehan et al., 2009). In this study we showed the antifungal activity of Virkon-S against *S. parasitica in vitro* and *in vivo*, which is the first time as we know of.

In the determination of minimum inhibitory concentration, Virkon-S inhibited germination of *S. parasitica* as low as 20 ppm. One interesting feature in this *in vitro* experiment is the appearance of bulged structure at the concentration where there was no germination or mycelial growth (Fig. 1). In the life cycle of *S. parasitica*, primary zoospores are released from sporangium at the end of hyphal cells. These zoospores are motile but swim only for a short time and form cyst that release a secondary zoospore

which are also motile for a longer period of time than primary zoospores and play impartment roles in dispersion and infection of *S. parasitica* (Van West, 2006). The motile primary and secondary zoospores have two flagella with different shape. Most of the structure observed in the samples treated with Virkon-S did not show the presence of these flagella. Inhibition of cyst germination by several chemicals has been reported from well-known plant pathogenic oomycetes, *Phytophthora infestance* (Matheron et al., 2000). So, it is possible that these bulged structures are cyst formed from the primary zoospores, but further investigation is necessary to explain the reason for the formation of this bulged structure.

Further *in vitro* assay of mycelial growth inhibition on PDA plate, the minimum concentration that required for the inhibition of spore germination and the resulting mycelial growth inhibition appeared as low as 4 ppm and partial (53.8%) inhibition was observed on a PDA plate containing 2ppm Virkon-S. Therefore, the minimum oomyceticidal concentration was determined as 4 ppm on PDA plates. When the inhibition of spore germination was repeated on thin PDA plates for enumeration, same results with complete spore germination inhibition at 4 ppm and 59% inhibition at 2 ppm was obtained. Also, bulged structures were observed at the concentration where there was no spore germination (Fig. 4).

In order to use Virkon-S for the control of saprolegniasis, it should be safe to fish or treated embryo. The cytotoxicity of Virkon-S was tested with EPC cell which was

originated from Fathead minnow, *Pimephales promelas.* No cytotoxic effect was observed at 10 and 100 ppm but there was slight decrease (97%) at 500 ppm (Fig. 5). I out experiment, the minimum inhibitory concentration was 10 ppm and even 4 ppm was enough to inhibit the spore germination and mycelial growth. Therefore, it seemed that Virkon-S could be used to control the growth of *S. parasitica* without any cytotoxic affect at the concentration which can inhibit the fungus. This was further confirmed by acute toxicity test. No toxic effect was observed from dishes kept at 2, 4, 10 ppm for 96 h (Fig. 6). Also, these fish did not show any after effect up to 40 days after termination of the test. Considering all this experiment, the maximum acceptable toxicant concentration (MATC) was determined as 10 ppm.

The efficacy of Virkon-S for the treatment was tested with fish that artificially infected with *S. parasitica*. Fishes wounded and inoculated with *S. parasitica* zoospores showed clear symptoms of saprolegniasis 10 days after inoculation, which was confirmed by microscopic observation and culture on PDA plates. Fishes treated with 2, 4 and 10 ppm Virkon-S sHd 50, 100%, recovery of the disease, respectively after 10 days' treatment. The concentrations required for the control of the disease coincide with the concentrations that were obtained from in vitro assay. In addition to 100% survival of the treated fish, all recovered fish showed regeneration of scales on wounded area, which is the indication of complete treatment.

Virkon-S is approved as quasi-drug that can be used as disinfectant for the treatment of aquaculture facilities and equipment.

V. Conclusion

The results in this study indicated that Virkon-S can be used for the control of saprolegniasis at the concentration of 4 ppm at which no toxic effect was observed in cultured fish cells or fish in tank. Although further research on the effect of human is necessary, Virkon-S can be a good candidate as a control agent of saprolegniasis that causes huge economic losses in aquaculture.

The animal protocol used in this study has been reviewed by the Pukyong National-Institutional Animal Care and Use Committee (PKNU-IACUC) on their ethical procedures and scientific care, and it has been approved (Approved Number PKNU-2017-01).

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