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Anti-oomycete Activity of Chloramine-T against *Saprolegnia* Species Isolated from Rainbow Trout

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Abstract

Saprolegnia is a genus of oomycetes which comprises important pathogens of salmon and trout species. There is re-emergence of *Saprolegnia* in the vulnerable fish species due to lack of effective agents against the microbes. Here, we report the isolation of *Saprolegnia* species from rainbow trout (*Oncorhynchus mykiss*) and its identification as *S. parasitica* and *S. australis* based on the nucleotide sequences of the internal transcribed spacer region. The isolates were used for evaluation of the anti-oomycete activity of chloramine T (CA-T), a commonly used drug against bacterial gill disease. In molecular docking, CA-T was found to interact with key proteins, host targeting protein, TKL protein kinase, and plasma membrane ATPase of *Saprolegnia* through hydrogen bond and hydrophobic interactions, which indicates the probable activity against the organism. *In vitro* experiments revealed that 400 mg/L of CA-T can kill *S. parasitica*, whereas a higher concentration of 600 mg/L was required to produce the similar effect in *S. australis*. The results indicate that CA-T is effective against *Saprolegnia,* but susceptibility is variable amongst different species. Overall, the findings highlight the anti-*Saprolegnia* activity of CA-T and the importance of correct identification of the causative agent to decide the optimum dose for judicious use of drug.

Introduction

Saprolegnia is a genus of oomycetes encountered in fish and aquatic environments. The genus contains some of the important pathogens that are responsible for the declining population of wild and farmed aquatic animals (Fernández-Benéitez *et al.,* 2008; Ibrahim *et al.,* 2022). The organism is usually saprophytic, but it frequently becomes pathogenic to fish when they are stressed, injured, or debilitated. These pathogens cause a disease known as saprolegniasis in fish, which is characterised by the presence of a white or grey tuft of hyphae at the site of infection, such as skin, gills, and fins. If the infection is not treated, hyphae invade the underlying tissues and blood vessels, leading to impaired osmoregulation, respiratory failure, lethargy, loss of equilibrium, and ultimately death of the infected fish (Tedesco *et al.,* 2019). The pathogens also infect unfertilised or dead eggs and clump them together due to the intertwined hyphae, which further extend to nearby healthy ones. Saprolegniasis is one of the major economic setbacks to the aquaculture industry and hence, has a deleterious effect on global fish production. The disease occurs most commonly when the water temperature drops suddenly during winter, leading to mass mortalities of fish, amphibians, and other lower aquatic vertebrates/invertebrates (van Den Berg *et al.,* 2013; Costa and Lopes, 2022; ElGamal *et al.,* 2023). *Saprolegnia* infection is also favoured by other factors like existing injury and primary infection that provides a suitable niche for the zoospores to colonise.

In the past, *Saprolegnia* infection used to be controlled successfully by using malachite green, but the drug has been banned for use on fish intended for human consumption due to its harmful effects (Sudova *et al.,* 2007). It has led to recrudescence of *Saprolegnia* infections, particularly by *S. parasitica* in catfish, salmon, and trout species (Almeida *et al*., 2009). *Saprolegnia* infection is not only restricted to salmonids; instead, it can also cause high mortality, reaching up to 100% in other fishes such as silver perch (*Bidyanus bidyanus*), striped catfish (*Pangasianodon hypophthalmus*), and different species of tilapia (Lightner *et al*., 1988; Read *et al*., 2007; Ravindra *et al*., 2022). Therefore, it is important to discover or identify suitable control measures against *Saprolegnia* infection, to prevent losses in aquaculture. Hence, many chemicals have been tested and used to control *Saprolegnia* infection, but success rates are not quite promising. Currently, formalin, boric acid, hydrogen peroxide, and bronopol are used, but none matches the efficacy of malachite green (Leal *et al.,* 2018; Tedesco *et al.,* 2018; Kumar *et al.,* 2020). So, several studies are being conducted to discover or develop an effective and safe drug against *Saprolegnia* that does not pose any additional stress to the fish. Amidst this, chemical oxidants are emerging as a good and efficient alternative since microbes do not develop resistance against oxidation over time (Bowker *et al.,* 2011; Leal *et al.,* 2018). One such oxidizing agent is chloramine T (CA-T), which has been approved in the U.S. for use in aquaculture to control bacterial gill disease.

CA-T is an active chlorine compound with proven antibacterial and antifungal activity due to its oxidative nature. Its hypochlorite moiety can destroy DNA via oxidation and disrupt essential biological processes, thus preventing the microbes from reproducing. It also has a potential for forming a chlorine layer around the extracellular matrix that has an immediate destructive impact on the microbial surface (Harris *et al.,* 2004; Gottardi and Nagl, 2005; McKeen, 2012; Ferreira *et al.,* 2017). It is also reported to have rapid microbicidal action with lower chances of resistance development and low bioaccumulation potential due to high water solubility and biodegradability (FDA, 2014; Schmidt *et al.,* 2007). Use of CA-T in aquaculture is approved by the FDA under the Investigational New Animal Drug (INAD) exemption to generate more information on drug efficacy and safety data (Haneke, 2012; Johnson and Bosworth, 2012). In aquaculture, CA-T has been used to treat columnaris disease, bacterial gill disease, trichodiniasis, and monogenean trematode infestations (Gaikowski *et al.,* 2004; Nayak and Gaonkar, 2022). Despite its wide use in aquaculture, information on the efficacy of CA-T against *Saprolegnia* is limited. Considering the re-emergence of *Saprolegnia* species in farmed fishes and the lack of effective control measures, the present study was undertaken to evaluate the activity of CA-T against the *Saprolegnia* species isolated from rainbow trout.

Materials and Methods

Isolation and morphological observation

Saprolegnia species were isolated from infected rainbow trout carrying white cotton-like mycelial growth at the site of infection. The fish were reared using underground water in a recirculation system under confined conditions without any mechanical or biofiltration system at our institute facility (29.3603° N, 79.5530° E). The water temperature was maintained between 14 and 18℃ and the flow rate was kept at 0.00005 m^3/s. The fish were at the advanced fry stage, weighing 2-2.5 g and stocked at a density of five fish per litre of water. The mycelium from the infected fish was collected with sterile forceps and rinsed with autoclaved water. The mycelia was inoculated on potato dextrose agar (PDA) supplemented with 500 mg/L of ampicillin and 200 mg/L of chloramphenicol (Parra-Laca *et al*., 2015; Songe *et al*., 2016) and incubated at 20±1℃. Subculturing of the isolates was done by transferring a piece of agar containing hyphae on to fresh PDA. For microscopic observation, a piece of agar containing hyphae was inoculated in sterile water in a six-well plate and incubated at 20±1℃. The plates were observed daily for up to 10 days for the development of any characteristic feature using an inverted microscope (Olympus CKX53), and the findings were compared with previous reports (Vega-Ramírez *et al*., 2013; Magray *et al*., 2018).

Molecular identification

The isolates were cultured in potato dextrose broth (PDB) by inoculating a piece of agar containing *Saprolegnia* hyphae. The inoculated culture was incubated at 20±1℃ for 7 days, and the cotton ball like mycelia were used for the isolation of genomic DNA by DNAzol (Invitrogen) following the instructions of the manufacturer. The purity and quantity of the isolated genomic DNA were determined by nanodrop spectrophotometer (Thermo Scientific). The genomic DNA was used as template for amplification of the internal transcribed spacer (ITS) region using universal primers, ITS1 and ITS4 (White *et al*., 1990). The primers were purchased from Eurofins Genomics, India. The sequences of the forward (ITS1) and reverse (ITS4) primers are 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively. The primers ITS1 and ITS4 have a GC content of 63.16% and 45.0%, respectively.

The melting temperatures (Tm) of the primers are 60.99°C and 55.25°C for ITS1 and ITS4, respectively. The lyophilised primers were resuspended in nuclease-free water to get a stock solution of 100 pmol/µl. The PCR reaction was carried out in 20 µl, which consisted of 10 µl of 2X master mix (NEB), 5 pmol of each primer, 10 ng genomic DNA, and nuclease-free water to make up the volume. The cycling conditions for amplification were

kept at 95°C for 2 min for initial denaturation followed by 35 cycles of denaturation, at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR products from two isolates exhibiting minor differences in their growth morphology, were selected for further analysis. They were purified using a commercial kit (Wizard® SV Gel and PCR Clean-Up System, Promega) and sequenced at Agrigenome Labs located in India. The sequences were analysed using Bioedit (Hall, 1999) and BLAST (basic local alignment search tool) and then submitted to NCBI GenBank. A molecular phylogenetic tree was constructed by the neighbour-joining method of MEGA (Molecular Evolutionary Genetics Analysis) version 11 using a bootstrap test of 1000 replicates (Felsenstein 1985; Saitou & Nei 1987; Tamura *et al*., 2021). The evolutionary distances were computed using the Kimura 2-parameter (Kimura, 1980).

Molecular Docking Analysis

In order to determine the interaction between CA-T and the vital proteins of *Saprolegnia*, molecular docking analysis was carried out. Structure of CA-T in SDF format was downloaded from Pubchem. Homology modelling of *S. parasitica* proteins, viz., tyrosine kinase-like (TKL) protein kinase and plasma membrane ATPase, was done through the multiple threading approach of I-Tasser (Yang and Zhang, 2015). In addition, a template of *S. parasitica* host targeting protein-1 was identified by the fold-based approach of pDOMTHREADER (Tandel *et al*., 2021). The template with high score was used for homology modelling using Modeller 9.18 (Sali and Blundell, 1993). The modelled 3-D structures were refined using ModRefiner (https://zhanglab.ccmb.med.umich.edu/ModRefiner/) and the quality was analysed using saves server [SAVESv6.0—Structure Validation Server (ucla.edu)]. The energy for binding of CA-T with the proteins was determined by AutoDock Vina (Trott and Olson, 2010). Among the predicted CA-T and protein complexes, the model with a higher negative docking score was selected to examine the hydrogen bonding and hydrophobic interactions (Bhat *et al*., 2020). Finally, the 2D and 3D structures were visualised by LigPlot 2.1 and PyMOL, respectively (Laskowski and Swindells, 2011; The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Determination of Minimum Inhibitory Concentration

CA-T powder was purchased from MP Biomedicals. A stock of 10,000 mg/L of CA-T was prepared using sterile distilled water and then filtered using a membrane filter. The working concentration was prepared by further diluting the stock solution in sterile distilled water. Minimum inhibitory concentration (MIC) of CA-T was determined against two species, *S. parasitica* and *S. australis,* by broth dilution method following the published protocol (Thakuria *et al*., 2022). Both the strains were first adapted to glucose yeast extract agar (GYA), and in the subsequent experiments, four day-old culture was used to maintain uniformity. Briefly, 400 µl of glucose yeast extract broth (GYB) containing different concentrations of CA-T (100, 200, 300, 400, 500, and 600 mg/L) was added to the designated wells of a 48-well plate. Then, an agar plug of ~4 mm diameter containing *Saprolegnia* hyphae was excised from the four day-old culture and added to each well. The experiment also included growth control wells containing only GYB and the agar plug and sterile control wells which contain only GYB and sterile water. For each concentration of CA-T, growth control, and sterile control, triplicate wells were kept. The plates were incubated at 20±1℃ for 48 h. After incubation, 100 µl resazurin (300 µM in 10 mM sodium phosphate buffer, pH 7.4) was added to each well and monitored visually for change in colour from blue to pink. Then, MIC was recorded as the minimum concentration of CA-T that prevented change in colour in the treated wells. The experiment was repeated three times.

Determination of Minimum Oomyceticidal Concentration

The minimum oomyceticidal concentration (MOC) of CA-T was determined by observing the presence or absence of hyphal growth following the published protocol (Thakuria *et al*., 2022). The *Saprolegnia* hyphae were treated with different concentrations of CA-T, as in the case of MIC. Similarly, growth control wells were also included in the experiment. After 48 h of incubation, the control and treated hyphae were shifted to a fresh GYA that does not contain CA-T. The plate was marked into four chambers on the back side, and the control as well as the treated hyphae were inoculated at the designated site and incubated for 24 h at 20±1℃. The plates were then examined for hyphal growth after incubation for 24 h. The minimum concentration of CA-T that inhibited visible hyphal growth of treated *Saprolegnia* on fresh GYA was recorded as MOC. The experiment was repeated three times.

Radial Growth Inhibition Assay

To determine the effect of CA-T on hyphal growth of *Saprolegnia*, a radial growth inhibition assay was performed following the published protocol with some modifications (Shin *et al.*, 2017). Briefly, equal portions of 2X GYA and 2X CA-T were mixed to obtain 1X GYA containing different concentrations of 100, 200, 500, 1000, 2000, and 4000 mg/L of CA-T. After mixing, 20 mL of each GYA/CA-T mixture were poured into 90 mm petri plates and allowed to solidify. For growth control, 2X GYA mixed with sterile distilled water in a 1:1 ratio were used. Then an agar plug of ~1 cm in diameter containing *Saprolegnia* hyphae was cut from a 4 day-old culture and inoculated onto the center of each GYA/CA-T plate. The

plates were incubated at 20±1℃, and radial growth of hyphae was recorded in diameter every 24 h till 72 h. Each concentration was tested in triplicates and repeated three times.

The percent inhibition of radial growth (PIRG) was calculated using the following formula:

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

where, R1=radial growth of control plate, and R2=radial growth of treatment plate.

Statistical Analysis

Data obtained from the radial growth inhibition assay were subjected to statistical analyses using GraphPad Prism 8.4.2. The significant difference of each parameter between the given groups was tested using the independent-samples t-test. In order to detect the possible interactions between the CA-T concentration and incubation period in each group, a one-way ANOVA test was used, and then a two-way ANOVA was applied to calculate the effect of the CA-T concentration and incubation period individually and by their interactions on the radial growth. The levels of statistical difference were set at $P<0.05$ (significant) and $P<0.01$ (very significant). Significant differences ($P<0.05$) between the different concentrations of the same incubation period are indicated with different letters (a–e). Very significant ($P<0.01$) differences between the effects of CA-T on different species are indicated by asterisk. The data were expressed as mean ± SEM.

Results

Morphological Observation

The isolates produced white hyphae on PDA, and the growth extends radially from the centre. At first, hyphae were found to penetrate into the agar and spread inside it. This could be observed when the plates were held against light. Following this, some hyphae started growing above the surface, which gave a white cotton-like morphology (Figure 1 A & E). Among the isolates, two different growth patterns were observed.

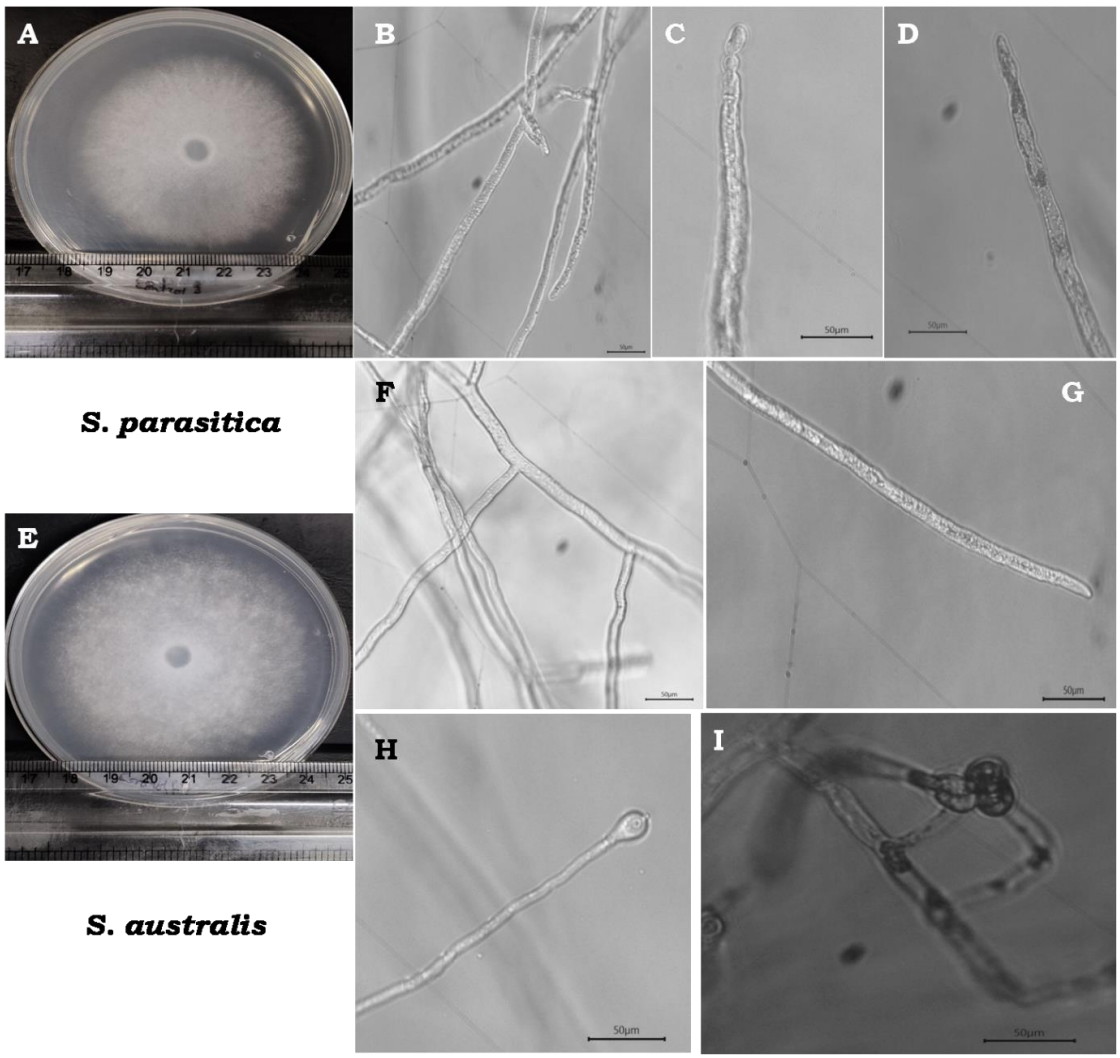

Figure 1. Growth and microscopic morphology of *Saprolegnia species.* 48 h old *S. parasitica* (A) and *S. australis* (E) culture on glucose yeast extract agar. Aseptate hyphae (B) and zoosporangium of *S. parasitica* (C & D). Aseptate hyphae (F), zoosporangium (G), immature archegonium (H) and antheridial branch wrapping around oogonium (G) of *S. australis*.

One isolate had the hyphae extending radially in a more uniform pattern on the agar, and the growth was comparatively slower. Whereas the other isolate had faster growth along with luxuriantly growing hyphae above the surface of agar. Microscopic observation revealed that both the isolates had aseptate hyphae with branches at some points (Figure 1 B & F). In both the isolates, cylindrical zoosporangia were observed at the tip of the hyphae (Figure 1 C, D & G). After 10 days of incubation in water, some sexual reproductive structures, like immature archegonium (Figure 1 H) and antheridial branch wrapping around oogonium (Figure 1 G), were observed in the isolate, which also showed faster growth, whereas no such features were observed in the other.

Molecular Identification of the Isolates

Amplification of the ITS region from the genomic DNA of the isolates using ITS1/ITS4 primers produced amplicons of approximately 750 bp. Nucleotide sequences from forward and reverse direction sequencing were analysed, and contigs of 706 bp and 705 bp were prepared. In BLAST, the contigs showed maximum identity with *S. parasitica* and *S. australis,* respectively. The nucleotide sequences were submitted to NCBI GenBank, and the accession numbers MT912581 and MT912582 were obtained. The phylogenetic relationship of the isolates with other *Saprolegnia* species was inferred using ITS sequences (Figure 2). Other species of *Saprolegnia,* such as *S. hypogona, S. ferax, S. diclina,* and *S. delica,* were also included in the analysis. In the phylogenetic tree, MT912581 formed a group with *S. parasitica* supported by a bootstrap value of 99. MT912582 formed a single group with *S. australis,* supported by a bootsrap value of 100. Based on the molecular analysis, the isolate with faster growth rate was identified as *S. australis* (MT912582) and the other as *S. parasitica* (MT912581).

***In-silico* Analysis**

In molecular docking, CA-T was found to bind with different proteins of *Saprolegnia* through a number of hydrophobic interactions and hydrogen bonding. The details of the interaction between CA-T and *Saprolegnia* proteins in 2D and 3D format are shown in Figure 3 and Table 1. In hydrophobic interactions, five residues of host targeting protein and seven residues each of plasma membrane ATPase and TKL protein kinase were involved. In addition to the hydrophobic interactions, one hydrogen bond each involving Ser89 and Ser248 in host targeting protein and plasma membrane ATPase, respectively, was observed. In the case of TKL protein kinase, two hydrogen bonds at Trp560 and Ser561 were observed. Among the proteins, the lowest binding energy, -6.5 Kcal mol^{-1}, was found to be in the interaction between CA-T and plasma membrane ATPase.

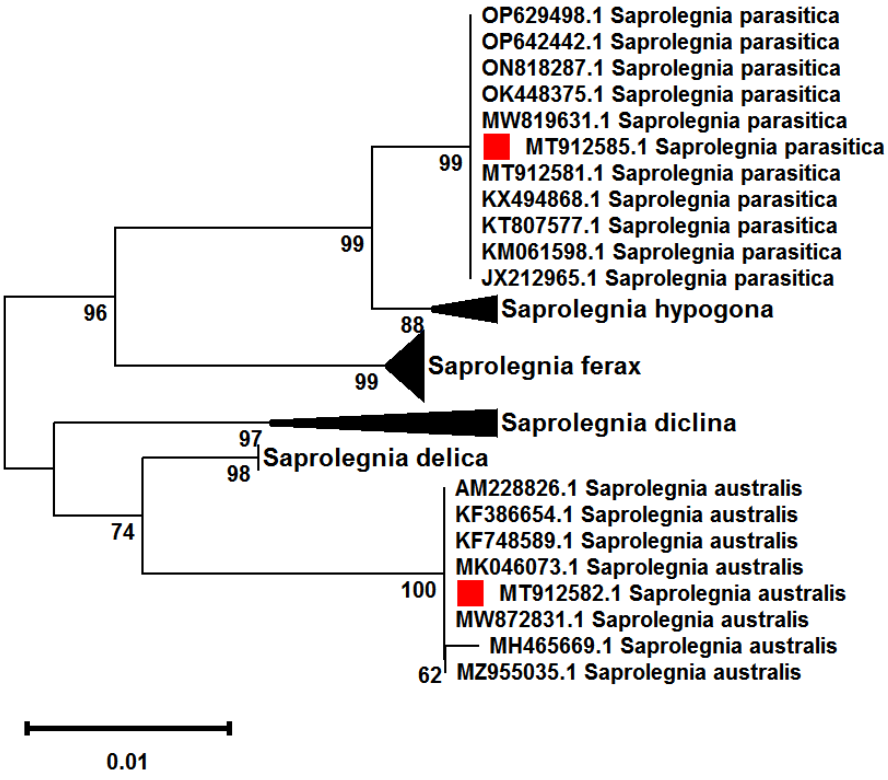

Figure 2. Phylogenetic relationship of the present isolates, *S. parasitica* and *S. australis* (red box) with other *Saprolegnia* species. The percentage of replicates in the bootstrap test is shown below the branches. Subtrees formed by the same species have been condensed and only the name is written.

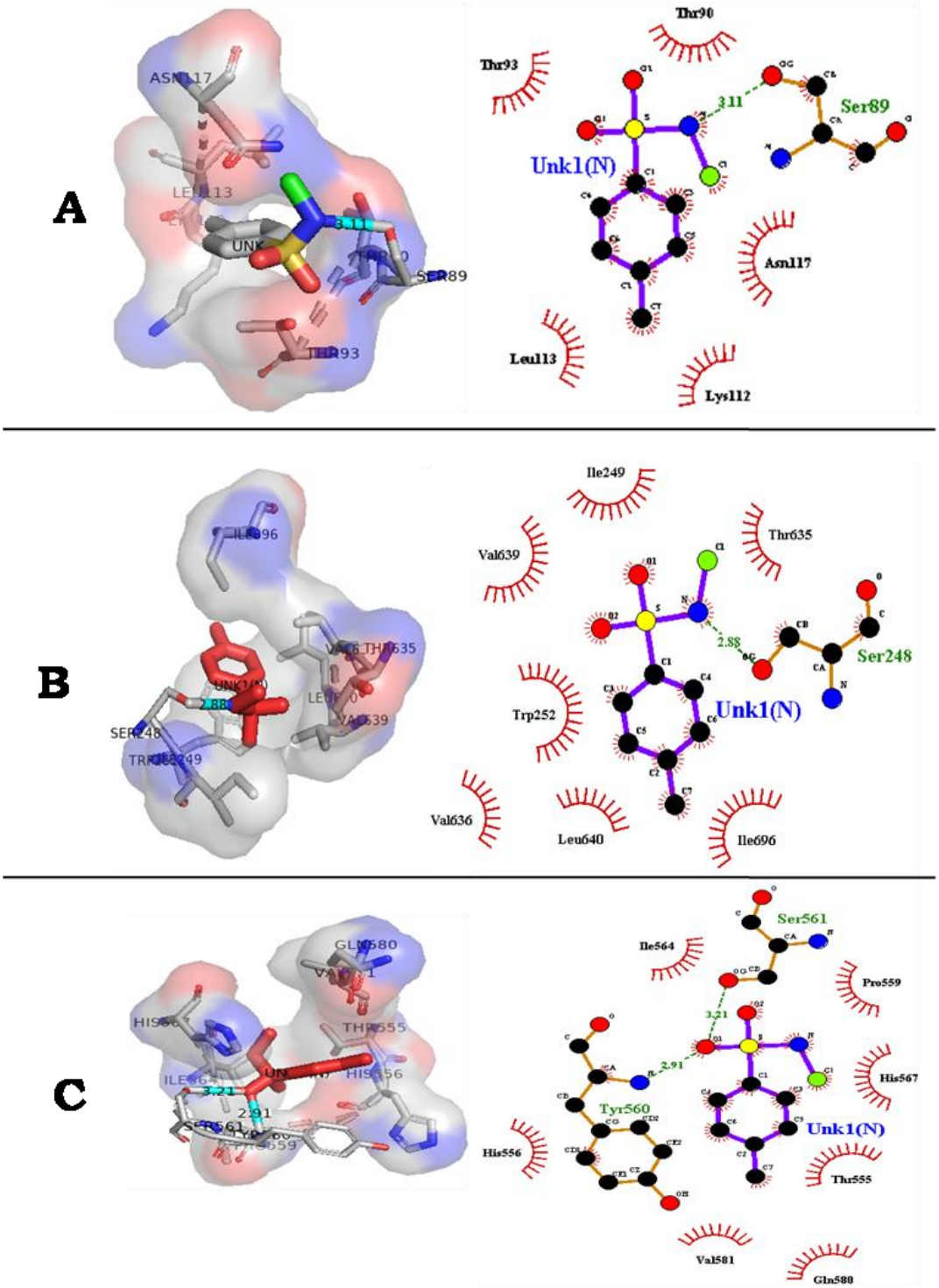

Figure 3. *In silico* analysis of potential interactions of Chloramine-T (CA-T) with proteins of *S. parasitica.* (A) CA-T-*S. parasitica* host targeting protein, (B) CA-T- plasma membrane ATPase (C) CA-T-TKL protein kinase in 2D and 3D format.

Table 1. Interaction of chloramine T (PubChem CID 3641960) with different proteins of *Saprolegnia*

Protein	Residues involved in hydrophobic interactions	Residues involved in hydrogen bonding and bond length (Å)	Total no. of hydrogen bond	Docking energy (kcal/mol)
Host targeting protein	Thr93, Thr90, Leu113, Lys112, Asn117	Ser89:3.11	1	-4.3
Plasma membrane ATPase	Ile249, Val639, Trp252, val636, Leu640, Ile696, Thr635	Ser248:2.88	1	-6.5
TKL protein kinase	Ile564, His556, Val581, Gln580, Thr555, His567, Pro559	Trp560: 2.91 Ser561:3.21	2	-6.3

Minimum Inhibitory and Minimum Oomyceticidal Concentration

CA-T exhibited its inhibitory effect against *S. parasitica* and *S. australis* at different concentrations. The MIC of CA-T, which arrested the growth and prevented the reduction of resazurin (blue) to resorufin (pink), was found to be 400 mg/L for *S. parasitica* and 500 mg/L for *S. australis* (Figure 4). *S. parasitica* hyphae treated with MIC and above concentrations of CA-T also showed no hyphal growth when shifted to fresh GYA, indicating its killing effect. In the case of *S. australis*, the concentration of CA-T to kill the hyphae was found to be more than its MIC. As shown in Figure 4, there was hyphal growth in *S. australis* treated with 500 mg/L but not at 600 mg/L. Based on the absence of hyphal growth, MOC was recorded as 400 mg/L and 600 mg/L for *S. parasitica* and *S. australis,* respectively.

Radial Growth Inhibition

The *Saprolegnia* species showed a dose-dependent rate of radial hyphal growth on GYA supplemented with different concentrations of CA-T. In *S. parasitica*, there was a gradual increase in radial growth diameter at control and 100 mg/L and reached the edges of the petri plate by 72 h. At 200 mg/L, the radial growth diameter was significantly less up to 48 h but was almost similar to control by 72 h. The radial growth at 500 mg/L was significantly ($P<0.05$) less than the control, 100 mg/L, and 200 mg/L. At 1000 mg/L, 2000 mg/L, and 4000 mg/L, there was no hyphal growth throughout the incubation period. In the same way, the percent inhibition on radial growth was significantly ($P<0.05$) different between 100 mg/L, 200 mg/L, and 500 mg/L of CA-T at 24 and 48 h. (Figure 5). A similar trend of reduced growth with increased concentration of CA-T was also observed in *S. australis*. Unlike *S. parasitica*, the radial hyphal growth was also observed at 1000 mg/L in *S. australis,* although the diameter was significantly ($P<0.05$) reduced. Likewise, the percent inhibition on radial growth was found to be significantly increased at successively higher concentrations of CA-T, reaching 100 % at 2000 mg/L and 4000 mg/L (Figure 6). The actual radial growth of hyphae was noted as the measured diameter minus 10 mm (the size of agar plug) for each plate. Further, the effect of CA-T on *S. parasitica* and *S. australis* was found to be very significantly ($P<0.01$) different at 500 mg/L and 1000 mg/L (Figure 7).

Discussion

In this study, we have evaluated the anti-oomycete activity of CA-T *in vitro* against two *Saprolegnia* species, *S. parasitica* and *S. australis,* isolated from rainbow trout. These two species have been reported as the common causative agent of saprolegniasis in salmonids (Hussein *et al*., 2001). During culture on agar media, two stages of hyphal growth were observed in both the isolates. At the initial stage, hyphae were found to

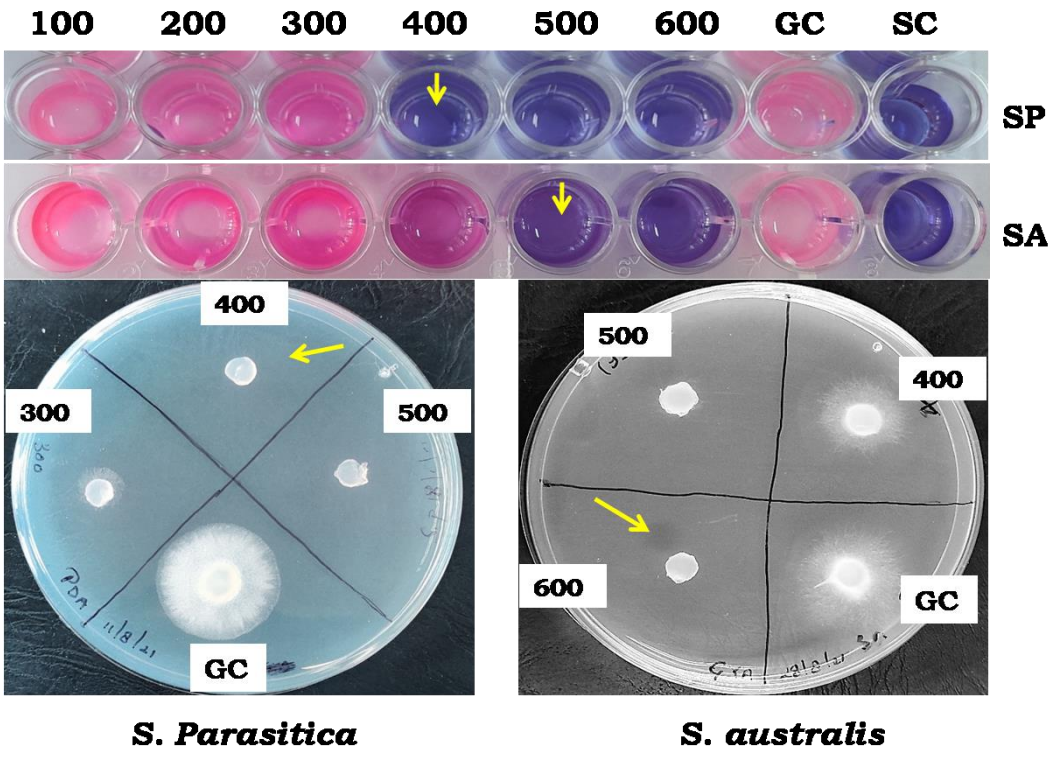

Figure 4. Minimum inhibitory concentration (MIC) and minimum oomeceticidal concentration (MOC) of CA-T against *Saprolegnia species.* SP- *S. parasitica*, SA- *S. australis*, GC- growth control, SC- sterile control, 100 to 600- different concentrations of CA-T in mgL^{-1}. The arrow in the above panel indicates MIC (purple wells) and the arrow in the agar plate indicates MOC (absence of hyphal growth). Figures shown represent 9 replicates.

ramify inside the agar medium, followed by growth above the surface, giving the appearance of white cotton. In *Saprolegnia,* intramatrical hyphae that penetrate into the substratum are responsible for anchoring the mycelium and absorption of nutrients. In contrast, the extramatrical hyphae are longer, grow above the surface, forming the visible portion of mycelium, and also carry the reproductive organs (Digamadulla *et al*., 2016). On microscopic observation, non-septate hyphae with rare branches were observed in both the isolates. The presence of non-septate hyphae is a characteristic of oomycetes, particularly *Saprolegnia* (Magray *et al*., 2019). After incubation for 10 days in water, one of the isolates developed sexual reproductive structures, but the other did not. Based on the morphology, the two species could be differentiated, but accurate identification was not possible. Our observations can be corroborated with previous reports that some isolates from fish lesions fail to develop sexual reproductive structures *in vitro,* making it difficult to identify at species level (Steuland *et al*., 2005; Die´guez-Uribeondo *et al*., 2007). Species

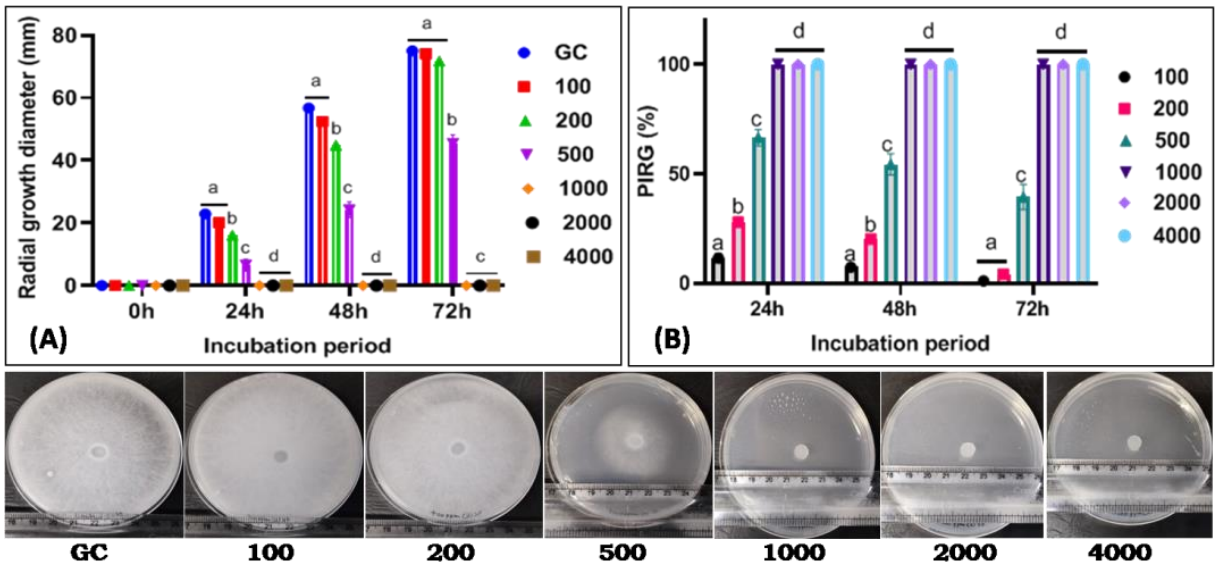

Figure 5. Effect of CA-T on radial growth of *Saprolegnia parasitica.* (A) The diameter of radial growth increases with time at GC (growth control), 100, 200 and 500 mgL^{-1} but not at higher concentrations. (B) There is 100% inhibition in radial growth at 1000, 2000 and 4000 mgL^{-1}. In the lower panel, radial hyphal growth on GYA containing different concentrations of CA-T at 72 h is shown. Significant differences (P<0.05) are indicated by different letters (a-d). Data are presented as mean ± SEM (n=9).

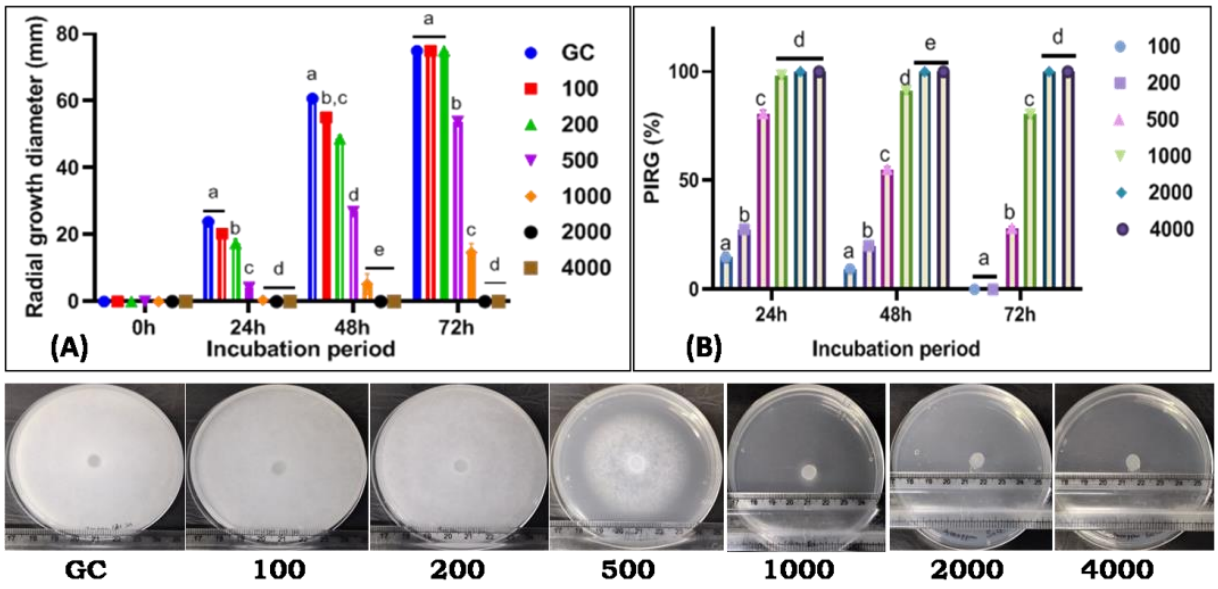

Figure 6. Effect of CA-T on radial growth of *Saprolegnia australis.* (A) The diameter of radial growth increases with time at GC (growth control), 100, 200, 500 and 1000 mgL^{-1} but not at higher concentrations. (B) There is 100% inhibition in radial growth at 2000 and 4000 mgL^{-1}. In the lower panel, radial hyphal growth on GYA containing different concentrations of CA-T at 72 h is shown. Significant differences (P<0.05) are indicated by different letters (a-e). Data are presented as mean ± SEM (n=9).

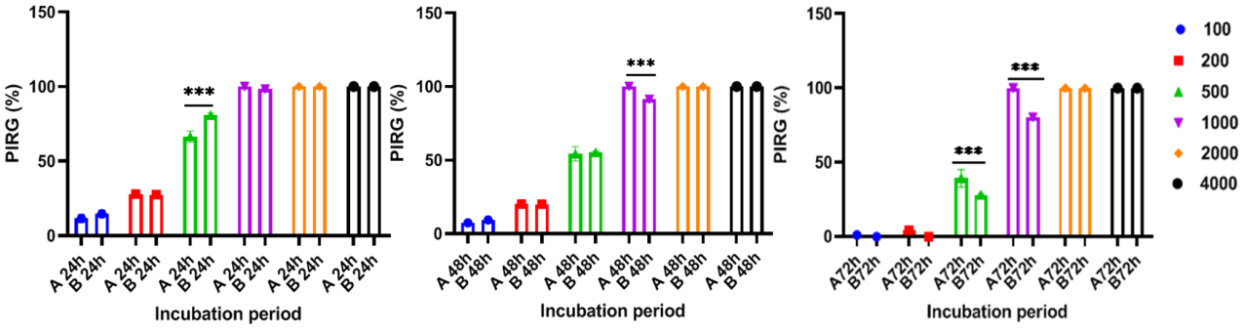

Figure 7. Comparison on the effect of CA-T on *S. parasitica* (A) and *S. australis* (B) at 24, 48 and 72 h. Very significant differences (P<0.01) are indicated by asterisk. Data are presented as mean ± SEM (n = 9).

identification is important to determine whether the disease is due to a pathogenic or saprophytic strain (Steuland *et al*., 2005)**.** It is also essential to decide the optimum dose of the anti-oomycete agent because different strains exhibit variable susceptibility (Thakuria *et al*., 2022). Since it was difficult to identify the species based on its morphology, molecular identification based on the nucleotide sequence of the ITS region was done. The ITS region is the most commonly targeted site of DNA and also the barcode accepted by the oomycete and mycology community for species identification (Robideau *et al*., 2011). The sequences MT912581 and MT912582 showed maximum identity with ITS-rDNA of *S. parasitica* and *S. australis,* respectively. Further, in molecular phylogeny, it was found that the current isolates are placed in two different clades. MT912581 formed a cluster along with *S. parasitica,* which is closely related to *S. hypogona,* whereas MT912582 clustered with *S. australis,* having *a* closer relation to *S. delica,* corroborating the previous report (Sarowar *et al*., 2019).

Similar to identification of the pathogens, selection of a suitable and potent anti-*Saprolegnia* agent is also equally important for judicious use of drugs. As there are no chemicals as effective as malachite green, a number of drugs have been tried for control of saprolegniasis. While some of the drugs lack efficacy, some are likely to be banned soon due to their adverse effect on the host, user, and/or environment (Magaraggia *et al*., 2006; Ali *et al*., 2014). Among the various compounds, CA-T is one that has been tried by a few researchers against *Saprolegnia* infection, but the detailed studies are still lacking (Ghazvini *et al*., 2012; Lahnsteiner, 2021). As compared to its use against bacterial infection, information on application of CA-T against *Saprolegnia* infection is limited. CA-T is a low toxic and mild oxidising agent that is being used worldwide as a disinfectant and antiseptic. This versatile synthetic compound is reported to have fungicidal, bactericidal, algicidal, virucidal, and germicidal activity (Nayak and Gaonkar, 2022). CA-T has been routinely used in aquaculture as treatment for bacterial gill diseases or external columnaris (Gaikowski *et al*., 2008). So, to proceed with the evaluation of its anti-*Saprolegnia* effect, first *in silico* analysis of the interactions between CA-T and vital proteins of *Saprolegnia* was carried out. In molecular docking, CA-T was found to interact with three key proteins, namely the host targeting protein, TKL protein kinase, and plasma membrane ATPase of *Saprolegnia* through hydrogen bonds and hydrophobic interactions. Molecular docking is widely used for predicting the binding affinity of a drug molecule with the protein or receptor of interest (Agu *et al*., 2023). Generally, the drug targets are cell membranes, proteins, enzymes, ion channels, etc that are essential for the metabolic and biosynthetic pathways of the microbe (Mazu *et al*., 2016). So, the host-targeting protein (htp) used for molecular docking in this study may prove to be a potential drug target because it is an effector molecule that translocates into the host cell by binding to tyrosin-O-sulphate, indicating a possible role in host-microbe interactions (Wawra *et al*., 2012)**.** Similarly, tyrosine kinase-like (TKL) protein kinases are widely distributed in oomycetes with several potential roles, for example, in growth, development, stress response, and especially virulence, and thus, useful targets for many drug discoveries (Cohen, 2002; Judelson and Ah-Fong, 2010; Shen *et al*., 2019). Plasma membrane ATPase is also a vital protein for maintaining electrochemical gradient across the membrane and also serves as a preferred target for antifungal drugs (Kjellerup *et al.,* 2017). Thus, the strong binding affinity of CA-T with these key proteins of *Saprolegnia* indicates that it may be an effective anti-oomycete agent. Further, to confirm the anti-*Saprolegnia* activity, *in vitro* experiments were carried out using *S. parasitica* and *S. australis*.

The MIC and MOC of CA-T against *S. parasitica* were found to be 400 mg/L, but higher concentrations were required to produce the similar effects in *S. australis*. The MIC and MOC of CA-T were less than the inhibitory concentration of boric acid, a known and patented agent for control of saprolegniasis (Ali *et al*., 2014). It was also much lesser than the effective concentration of NaCl, i.e., 15 g/L (Marking *et al*., 1994; Das *et al*., 2012). The difference in MIC and MOC between *S. parasitica* and *S. australis* indicates variation in drug susceptibility, which has also been observed in our previous study (Thakuria *et al*., 2022). In other experiments also, it was observed that MIC and MOC are

usually higher in *S. australis* as compared to *S. parasitica* (unpublished data). This may be due to the difference in the cell wall or membrane component among the species (Escriba, 2008). It may also be possible that *S. australis* is naturally more tolerant to anti-oomycete compounds or has undergone mutations that provide them resistance (Blum *et al*., 2012; Rezinciuc *et al.,* 2014)**.** The inhibitory effect of CA-T was also determined through a radial growth inhibition assay, a common and reliable method to measure the effect of a drug on hyphal growth (Brancato and Golding, 1953; Hendricks *et al*., 2017; Ebadzadsahrai *et al*., 2020). The percent inhibition in radial growth was determined by comparing the diameter of the test group to that of the control. At higher concentrations, there is complete inhibition of hyphal growth, and PIRG remains 100% throughout the experiment. At lower concentrations, the PIRG is higher at 24 h as compared to 72 h. It is because the growth of the inoculums was arrested initially to some extent and then started growing slowly with time. However, at 500 mg/L, the radial growth was significantly reduced in both species and the difference from the control and other lower concentrations of CA-T was apparent. At 1000 mg/L, little hyphal growth was observed in *S. australis* after incubation for 48 h but not in *S. parasitica*. This means that CA-T can effectively inhibit the growth of *Saprolegnia* species, including the more tolerant strains. Overall, this study has highlighted the importance of correct identification of pathogens, applications of *in silico* analysis in drug discovery, and most importantly, the anti-oomycete effect of CA-T against *Saprolegnia*. Future works may be focused on evaluation of *in vivo* efficacy of CA-T in disinfection of eggs and treatment of saprolegniasis in infected fish. Considering the effective oomyceticidal effect against *Saprolegnia* as observed in this study and low bioaccumulation potential due to high water solubility and biodegradability as reported earlier and easy availability, CA-T may become a popular and effective anti-*Saprolegnia* agent for management of saprolegniasis in fish farms.

Ethical Statement

No genetically engineered organisms and experimental fish were used in this study. All the methodologies followed in this study were approved by Institutional Research Committee of ICAR-Directorate of Coldwater Fisheries Research, Bhimtal.

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Author Contribution

Vinita Pant - maintenance of the *Saprolegnia* isolates, anti-*Saprolegnia* assay, writing manuscript, Victoria C. Khangembam and Dimpal Thakuria - concept and design of experiments, isolation and identification of *Saprolegnia* species, anti-*Saprolegnia* assay, writing manuscript, Raja Aadil Hussain Bhat-molecular docking and statistical analysis, Nityanand Pandey-sample collection and writing manuscript, Amit Pande and Pramod Kumar Pandey-manuscript editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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