Iranian Journal of Fisheries Sciences 22(6) 1305-1317 2023
DOI: 10.22092/ijfs.2024.130781

Short Communication

Inhibitory effects of the Iranian propolis ethanolic extract on different life stages of two *Saprolegnia parasitica* isolates recovered from rainbow trout (*Oncorhynchus mykiss*) eggs

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Keywords: *Saprolegnia parasitica*, Propolis, Anti-oomycete activity, Rainbow trout

Received: September 2023 Accepted: October 2023

Introduction

The global aquaculture industry has experienced dramatic growth since the beginning of the 21st century (5.3% per year). It now accounts for producing more than half of the amount of fish and other aquatic animals available for human consumption (FAO, 2022). This rapid increase in aquaculture production has spread several infectious diseases among wild and farmed aquatic species (Soltani *et al*., 2021; Ahmadivand *et al*., 2021; Tedesco *et al*., 2021). Notably, water molds of the Saprolegniaceae family, known as the causative agents of saprolegniasis, have caused significant losses in the freshwater aquatic species around the world, such as channel catfish (*Ictalurus punctatus*) in the USA (Bly *et al*., 1992), Coho salmon (*Oncorhynchus kisutch*) in Japan (Hatai and Hoshiai, 1992), rainbow trout (*Oncorhynchus mykiss*) and Persian sturgeon (*Acipencer persicus*) in Iran (Ghiasi *et al*., 2010; Shahbazian *et al*., 2010), angelfish (*Pterophyllum scalare*) in Egypt (Eissa *et al*., 2013), salmonid species in Chile and Lithuania (Sandoval-Sierra *et al*., 2014; Markovkaja *et al*., 2023), and *Pangasianodon hypophthalmus* in India (Kumar *et al*., 2022). Thus, exploring effective drugs to prevent and treat saprolegniasis is a high priority in the aquaculture industry.

For a long time, malachite green (MG) was used as an effective fungicide for treating saprolegniasis in aquaculture (Minor *et al*., 2014). However, many

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countries worldwide have prohibited using MG in aquaculture due to its highly genotoxic effects (Deutsch *et al*., 2023). Some treatments are used to control saprolegniasis, but they are not a suitable alternative. For example, the bath treatment of trout eggs with formalin is controversial because it poses health risks such as hypersensitivity and lung damage to fish farmers and consumers (Noga, 2010; Deutsch *et al*., 2023). Therefore, to manage saprolegniasis outbreaks, many researchers have focused on more environmentally acceptable compounds as an alternative to MG and other chemicals (Ali *et al*., 2019; Tedesco *et al*., 2019; Deutsch *et al*., 2023). For instance, various plant extracts have been screened against the mycelium of fish pathogenic *Saprolegnia* spp. (Cao *et al*., 2014; Emara *et al*., 2020; Shah *et al*., 2021; Meneses *et al*., 2022), and recently, the use of natural substances as a substitute for MG in aquaculture has been reviewed thoroughly by He *et al*. (2023).

Propolis is a natural resinous mixture of various plant exudates, wax, pollen, and essential oils (Zulhendri *et al*., 2021). It is collected and processed by different bee species, including honeybees, and it is applied as a protectant and disinfectant in their hives (Alvear *et al*., 2021). Its chemical composition varies significantly between geographical locations and plant sources from which it originates (Fernández-Calderón *et al*., 2020). There have been numerous reports on its beneficial health properties in aquaculture, such as growth-promoting, immunostimulatory, antibacterial, and antifungal effects (De la Cruz-Cervantes *et al*., 2018; Passão *et al*., 2023). For example, rainbow trout fingerlings fed with different dietary levels of ethanolic extract of propolis showed enhanced growth performance and improved plasma immune parameters such as lysozyme and superoxide dismutase activity (Deng *et al*., 2011). Moreover, there are some reports about the anti-oomycete activity of propolis on the fish pathogenic oomycete spp. such as *Aphanomyces invadanes* (Campbell *et al*., 2001), *A. astaci*, and *S. parasitica* (Miljanović *et al*., 2022). However, owing to its various chemical composition depending on the geographical and plant origin (Fernández-Calderón *et al*., 2020), propolis activity on micro-organisms can be unpredictable (Papp *et al*., 2021). Until now, there have been no reports on the anti-*Saprolegnia* activity of propolis from Iran.

In the present study, we aimed to investigate the *in vitro* anti-oomycete activity of Iranian propolis on various developmental stages (mycelia, zoosporangium, and zoospores) of two *S. parasitica* isolates recovered from infected rainbow trout eggs.

Materials and methods

Preparation of propolis extract

Propolis was obtained from the western part of Iran, mixed with 70% ethanol, and kept in a dark place with intermittent shaking for one week. The sample-to-

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solvent ratio was 1:10 (mass per volume). After that, the supernatant was filtered through a Whatman No. 1 filter paper (Whatman Ltd., England). The extracts were concentrated at 55°C and maintained in a closed, dark container (Mirmazloomi *et al*., 2022a).

In vitro assays

Saprolegnia isolates

Two isolates of *Saprolegnia parasitica* were recovered from infected rainbow trout eggs and used for this study. Briefly, the infected eggs were collected from a trout hatchery in the north of Iran and inoculated in sterile plates of yeast extract glucose chloramphenicol (YGC) agar and incubated at 18°C for 4-5 days. Morphological and molecular identification of the isolates were performed and finally, the nucleotide sequence of the isolates was submitted to the gene bank. The isolates were assigned as *S. parasitica* (Isolate KMG3, Accession number: MW819780) and *S. salmonis* (Isolate KMG2, Accession number: MW819740) (Mirmazloomi *et al*., 2022b). Purified cultures were kept on Sabouraud Dextrose Agar (SDA) and re-inoculated monthly. The inoculum was obtained by cutting the advancing edge of the young colonies (3d incubation at 18°C) using a sterile scalpel.

Concentrations of propolis tested (the HeMP method)

Propolis was diluted to the desired concentration in SDW. It was initially tested at 1000, 5000, 10000, 25000, 50000, and 100000 mg/L to determine the minimum inhibitory concentration (MIC) intervals following the HeMP method described by Stueland *et al*. (2005). Briefly, mycelia were grown on hemp seeds for 2 d at 18°C. Then, the *Saprolegnia*-colonized hemp seeds were transferred with sterile forceps into the wells of a 48-well flat-bottom tissue culture plate (NEST, China), and 1 mL of each test extract was added. SDW without extract and malachite green at 10 mg/L were set as negative and positive controls, respectively. The wells on the tissue culture plates were examined using a stereomicroscope (Olympus, Japan) 48 hours after the start of the exposure. The mycelial growth of *Saprolegnia* was graded as 0 for complete inhibition of the growth and 1 for partial or full growth of the mycelium on the hemp seeds (Fig. 1). The MIC value was expressed as the lowest concentration, at which fungal growth was not noticeable. All concentrations were tested four times. Once the concentration range, 0-1000 mg/L, was identified to stop *S. parasitica* hyphal growth, the test interval was narrowed down and was applied for further tests.

Effect of propolis on the growth of Saprolegnia mycelium

The propolis extract was added at 0, 10, 100, 250, and 500 mg/L to sterilize molten SDA held at 45°C. Then, the plates were inoculated with *Saprolegnia*-colonized agar plugs (5 mm), and the mean radial growth of

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Saprolegnia hyphae was calculated after 3d of incubation at 18°C. Each isolate was tested in triplicate for each concentration (Ali *et al*., 2019).

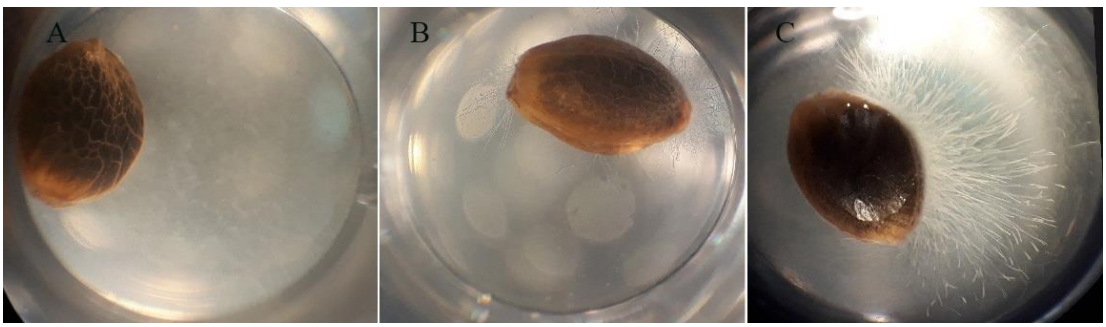

Figure 1: The mycelial growth of *Saprolegnia* was graded as 0 for complete inhibition of the growth (A) and 1 for a partial (B) or full growth of the mycelium on the hemp seeds (C).

Effect of propolis on the sporulation activity of Saprolegnia sporangia

Hemp seeds with 48 h actively growing mycelia (in SDW) were incubated with different propolis concentrations (200, 400, and 800 mg/L) and in SDW as a non-treated control for 90 min at 18°C. Then, they were washed three times with SDW, and the numbers of developed *Saprolegnia* sporangia were counted after 24 h of incubation in 12 mL SDW at 18°C. The mean of sporangia numbers was calculated based on three replicates per concentration. Moreover, the ability of *Saprolegnia* sporangia to discharge the zoospores was evaluated by light microscopy. Each isolate was tested in triplicate for each concentration (Ali *et al*., 2014).

Viability of treated Saprolegnia spores post-treatment

Zoospore suspension of *Saprolegnia* isolates was prepared according to (Ke *et al*., 2009). Firstly, mycelia were grown on hemp seeds for 3 d at 18°C. Then, the *Saprolegnia*-colonized hemp seeds were transferred to sterile plastic Petri dishes containing 12 mL SDW and incubated for 48 h at 18°C to induce sporulation. Finally, the swimming zoospores were harvested and maintained at approximately 2×10^4 mL^{-1} for the study. The propolis anti-oomycete activity on the zoospores was performed following the microdilution method described by Shah *et al*. (2021) with some modifications. Seven concentrations of the propolis extract and one dose of Formalin (Merck, Germany) were dissolved in 30% Glucose Yeast Extract (GY) broth, and 500 µL of each concentration was added to the eight columns of a 48-well flat-bottom tissue culture plate. After that, 500 µL of zoospore suspension was added to all wells to obtain 0, 6.25, 12.5, 25, 50, 100, and 200 mg/L of the extract and 250 mg/L formalin, respectively. Spore suspension and formalin served as negative and positive controls, respectively. All concentrations were tested thrice and in duplicates for each isolate (6 replicates per isolate and concentration). The plates were incubated for 24 h at 18°C. The

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Minimum inhibitory concentration (MIC) at which the optical microscopy detected no visible cyst germination was recorded. The results were confirmed by sub-culturing 10 µL of spore suspension from each concentration in YGC (yeast extract glucose chloramphenicol) agar and incubation at 18°C for 96 h. The experiment was performed in three internal and two external replicates (6 replicates per isolate and concentration).

Statistical analysis

One-way ANOVA followed by Duncan's comparison test was performed using SPSS version 15 (Microsoft Corporation, USA) for differences in radial growth of *Saprolegnia* and the number of *Saprolegnia* sporangia developed in different propolis extract concentrations. The differences between treated samples and controls were evaluated at a *p-value*<0.05.

Results and discussion

In the current study, we obtained the MIC value of 1000 mg/L for the ethanolic extract of Iranian propolis against *S. parasitica* in the HeMP method, indicating its anti-oomycete properties. Similarly, Campbell *et al*. (2001) showed good inhibitory activity of propolis (MIC=1000 mg/L) against the fish pathogenic oomycete, *Aphanomyces invadans*. Also, Pazin *et al*. (2019) found that Brazilian propolis and its identified compound, Artepillin C, could suppress the growth of the plant pathogenic oomycete, *Pythium aphanidermatum*, at MIC of 750 mg/L. These findings suggest that despite the variability in the origin of propolis, its bioactivity is always similar and remarkable (Miljanović *et al*., 2022).

Regarding the agar dilution method, propolis affected the mycelial growth of two *S. parasitica* isolates dose-dependently. In this regard, 100% growth inhibition was achieved at 250 and 500 mg/L for KMG2 and KMG3, respectively. Exposure to the lowest concentration of propolis (10 mg/L) caused an incomplete but statistically significant (p<0.05) inhibition of KMG3 (but not KMG2) hyphal growth. Treatment with 100 mg/L propolis gave a reduction of 52.6 and 50.6% for KMG3 and KMG2, respectively (p<0.05) (Fig. 2). These findings are in line with the study of Miljanović *et al*. (2022), who reported similar inhibition of *S. parasitica* mycelial growth by an effective concentration (EC50) value of Croatian propolis up to 206.60 mg/L. Such different sensitivity in the same species might be related to sub-culturing performed to maintain the *Saprolegnia* isolates. These continuous transfers are believed to change some physiological and genetic capacities (Eszterbauer *et al*., 2020). Moreover, some study shows the genetic diversity and the phenotype variability of the different *S. parasitica* strains (Ravasi *et al*., 2018), that is also evident in our results.

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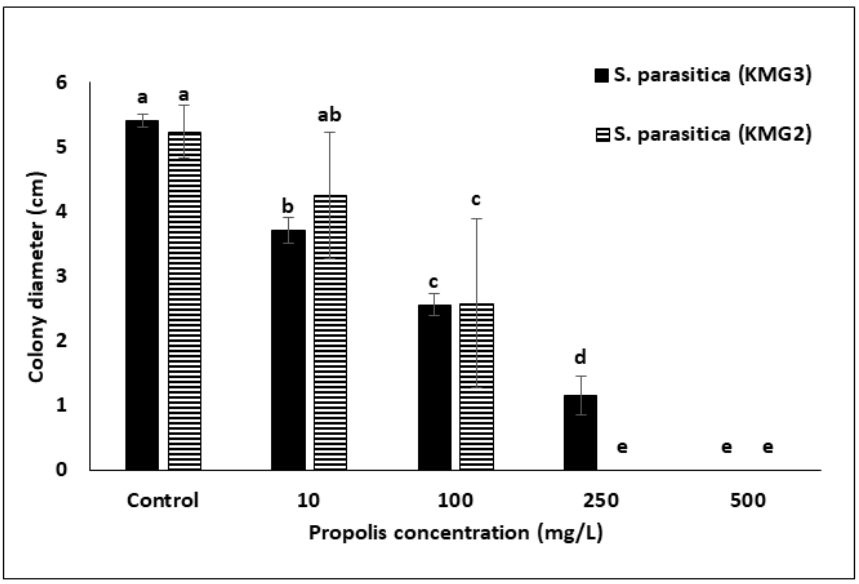

Figure 2: Inhibition of *Saprolegnia parasitica* (isolate KMG3) and *Saprolegnia parasitica* (isolate KMG2) growth on Sabouraud dextrose agar (SDA) following treatment with different propolis ethanolic extracts concentrations at 18°C. Different superscript letters indicate the existence of statistical differences among groups ($p<0.05$).

Saprolegnia sporangia, as well as their biflagellate zoospores, play an essential role in the infection process (Magray *et al*., 2019). For this reason, we chose these asexual reproduction compartments in SDW culture medium for further anti-oomycete assays to reach a concentration that could be applicable under field conditions, i.e., freshwater hatcheries in the trout farm (Stueland *et al*., 2005). Regarding the sporulation activity of *Saprolegnia* sporangia, it appeared to be stopped entirely during the first 24 h, following 90 min exposure to 800 mg/L propolis extract (Table 1); during this time, the entire zoosporangia of both *Saprolegnia* isolates became condensed (data not shown).

Table 1: Effect of propolis on the sporulation of *Saprolegnia* sporangia. The mean sporangia numbers (mean±SEM per 12 mL Sterile Distilled Water) were calculated based on 3 replicates per concentration. Different superscript letters indicate the existence of statistical differences among groups ($p<0.05$).

Tested propolis concentrations (mg/L)	*S. parasitica* (isolate KMG3)		*S. parasitica* (isolate KMG2)	
	The number of developed *Saprolegnia* sporangia	The ability of *Saprolegnia* sporangia to discharge the zoospores	The number of developed *Saprolegnia* sporangia	The ability of *Saprolegnia* sporangia to discharge the zoospores
0	43.3 ± 6.6^{a}	+	41.3 ± 5.9^{a}	+
200	39 ± 11^{a}	+	34.6 ± 15.3^{a}	+
400	28 ± 11.2^{ab}	+	19.6 ± 9.6^{ab}	+
800	1 ± 1^{b}	-	0^{b}	-

However, the concentration was not fully lethal, and the new sporangia continued growing and sporulating, 48 h after the exposure. A similar finding was

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observed for Boric Acid (BA) when it inhibited the formation of *Saprolegnia* sporangia and sporulation activity at 400 and 1000 mg/L, respectively (Ali *et al*., 2014). Nevertheless, compared to the sporulation activity, zoospore germination condition and viability were slightly different in our study, as they were inhibited after 24 h exposure to 200 mg/L propolis (Table 2). In the same way, Campbell *et al*. (2001) found that propolis resin solubilized in ethanol prevented zoospore motility of *A. invadans* at 10 mg/L. Furthermore, Miljanović *et al*. (2022) showed that fluid propolis preparations could affect zoospore motility and germination of *S. parasitica* at 61.88 and 23.62 mg/L, respectively. This wide range of inhibitory concentrations is probably related to oomycete species various propolis preparations, and experimental designs (Miljanović *et al*., 2022).

Table 2: The viability* of *Saprolegnia* spores after exposure to different propolis concentrations and Formalin (250 mg/L) for 24 h at 18°C.

Tested propolis concentrations (mg/l)	*S. parasitica* (isolate KMG3)	*S. parasitica* (isolate KMG2)
0	+	+
6.25	+	+
12.5	+	+
25	+	+
50	+	+
100	+	+
200	-	-
Formalin (250 mg/L)	-	-

+: Germination and growth of *Saprolegnia* cysts in yeast extract glucose chloramphenicol (YGC) agar.
- : No Germination and growth of *Saprolegnia* cysts in YGC agar.
* 10 µL of cysts suspension from each concentration was inoculated in YGC agar and incubated at 18°C for 96 h. The test was performed in three internal and two external replicates.

In the present work, the zoospores of the two *S. parasitica* isolates were also more susceptible to propolis than their mycelia (including mature zoosporangium). Correspondingly, Miljanović *et al*. (2022) reported that propolis could inhibit the hyphal growth of *S. parasitica* at 206.6 mg/L, while the required concentration for the inhibition of zoospore germination was 19.01 mg/L. Also, it was found that the ethanolic extract of *Thymus linearis* could arrest the hyphal growth of *S. parasitica* completely at 5120 mg/L, compared to the control group. In comparison, zoospore reduction occurred at 320 mg/L (Shah *et al*., 2021). Contrary to these findings, Madrid *et al*. (2015) showed more potent anti-oomycetes activity of *Laureliopsis philippianna* essential oils on *S. parasitica* and *S. australis* mycelia (MIC = 30 mg/L) than their zoospores (minimum fungicidal concentration (MFC=50 mg/L). Further, based on Miljanović *et al*. (2022) work, propolis could inhibit the hyphal growth of *A. astaci* at 8.59 mg/L, while the concentration needed to inhibit zoospore motility and germination was 154.68 and 19.52 mg/L, respectively. These variabilities in the susceptibility of

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different stages of the life of oomycete species could be related to the target proteins expressed in their mycelium/zoospores. For example, the protein thrombospondin, primarily expressed in *S. parasitica* cysts, is shown to be targeted by some flavonoids in propolis, and it could explain the sensitivity of zoospores than the mycelia (Miljanović *et al*., 2022).

Generally, propolis chemical composition is featured by certain pharmacological active ingredients regardless of its geographical origin (Fernández-Calderón *et al*., 2020; Passão *et al*., 2023). These components include flavonoids, phenolic acids, and aromatics (Papp *et al*., 2021; Passão *et al*., 2023). According to some recent studies, the antifungal activity of propolis could have originated from flavonoids such as pinocembrin and chrysin (Peng *et al*., 2012; Miljanović *et al*., 2022). For instance, pinocembrin, an important flavonone in propolis, was found to arrest *Penicillium italicum* hyphal growth by disrupting the fungus energy homeostasis and cell membrane integrity (Peng *et al*., 2012). Recently, based on molecular docking, flavonoids including apigenin, chrysin, and pinocembrin were identified to target *A. astaci* endochitinase, an enzyme involved in the degradation of the chitin layer in crayfish during the crayfish plague disease process (Miljanović *et al*., 2022). These flavonoids are typical of the poplar-type propolis whose plant of origin, mainly *Populus* trees, is also found in western Iran (Alimohamadi *et al*., 2012), the source of the propolis for this study. In the present study, we observed an anti-oomycete activity of Iranian propolis on two *Saprolegnia* isolates comparable to other studies (Campbell *et al*., 2001; Miljanović *et al*., 2022), despite the possible variation in their chemical compositions.

In conclusion, the propolis extract used in this study could be an effective and environmentally friendly substance to treat and prevent saprolegniasis in freshwater aquaculture. However, additional investigations are required to uncover the chemical composition of propolis samples from different regions of Iran, which should be paramount in large-scale aquaculture production. Moreover, an in vivo trial could be performed regarding the safety and applicability of this natural compound.

Acknowledgements

This work was performed at the Caspian Sea Ecology Research Center, Sari, Iran and the Faculty of Veterinary Medicine, University of Tehran, Iran.

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