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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Molecular identification of *Saprolegnia ferax* isolated from rainbow trout eggs in Turkey and antifungal activity of *Hypericum perforatum* and *Zingiber officinale* essential oils

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Abstract: Saprolegniales is an order of oomycetes, and their life is completely dependent on water, hence, they are often called "water molds". This order has both pathogenic and saprophytic lifestyles. Saprolegniasis is one of the biggest problems for the aquaculture industry, especially in hatcheries for rainbow trout (*Oncorhynchus mykiss*) production. Malachite green was successfully used to control *Saprolegnia* infection. However, Saprolegniasis has become a serious problem on aquaculture after its ban on the EU due to its toxicity and carcinogenic effects. In this study, the pathogenic fungus *Saprolegnia ferax* isolated from rainbow trout (*Oncorhynchus mykiss*) eggs was identified using molecular methods for the first time in Turkey. In addition, the susceptibility of the identified *S. ferax* strain to essential oils of *Hypericum perforatum* (St. John's wort) and *Zingiber officinale* (ginger) was determined. DNA barcoding was performed with ITS1-ITS4 primers for molecular identification of the isolated fungal species. DNA sequences obtained from the NCBI GenBank were used to construct a phylogenetic tree. The disk diffusion and tube dilution methods were used to determine the antifungal activities of essential oils. While ginger essential oil showed antifungal activity with a Minimum Inhibitory Concentration (MIC) of 15.62 µl/ml and Minimum Lethal Concentration (MLC) of 250 µl/ml, the MIC value of St. John's wort essential oil was found to be 125 µl/ml, the MLC value was 1000 µl/ml. The findings indicate that ginger essential oil may be considered an effective natural antifungal agent against *S. ferax*.

Keywords: Antifungal activity, disc diffusion, ITS region, medicinal herbs, tube dilution.

Türkiye'de gökkuşağı alabalığı yumurtalarından izole edilen *Saprolegnia ferax*'ın moleküler teşhisi ve *Hypericum perforatum* ve *Zingiber officinale* uçucu yağlarının antifungal aktivitesi

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Öz: Saprolegniales, suya tamamen bağımlı olarak yaşayan bir oomycetes (yumurta mantarları) takımıdır ve bu nedenle genellikle "su küfleri" olarak adlandırılırlar. Bu takım, hem patojenik (hastalığa neden olan) hem de saprofitik (çürükçül) yaşam tarzları geliştirmiştir. *Saprolegnia* enfeksiyonları (Saprolegniasis), özellikle gökkuşağı alabalığı (*Oncorhynchus mykiss*) üretimi yapılan kuluçkahanelerde, su ürünleri endüstrisi için en büyük problemlerden biridir. *Saprolegnia* enfeksiyonunu kontrol altına almak için malaşit yeşili başarıyla kullanılmıştır. Ancak, toksik ve kanserojen etkileri nedeniyle Avrupa Birliği'nde yasaklanmasının ardından Saprolegniasis ciddi bir sorun haline gelmiştir. Bu çalışmada gökkuşağı alabalığı (*Oncorhynchus mykiss*) yumurtalarından izole edilen *Saprolegnia ferax* türü patojenik mantar türü Türkiye'de ilk defa moleküler yöntemlerle teşhis edilmiştir. Ayrıca çalışmada, teşhis edilen *S. ferax* suşunun *Hypericum perforatum* (kantaron) ve *Zingiber officinale* (zencefil) uçucu yağlarına karşı duyarlılığı belirlenmiştir. İzole edilen mantar türünün moleküler teşhisi amacıyla ITS1-ITS4 primerleri ile DNA barkotlama yapılmıştır. Filogenetik analiz yapılması amacıyla NCBI genbank veri tabanından temin edilen DNA dizileri filogenetik ağaç oluşturulması amacıyla kullanılmıştır. Uçucu yağların antifungal aktivitelerinin belirlenmesi amacıyla disk difüzyon ve tüp dilüsyon yöntemleri kullanılmıştır. Zencefil uçucu yağı, 15,62 µl/ml'lik Minimum İnhibitör Konsantrasyonu (MIC) ve 250 µl/ml'lik Minimum Letal Konsantrasyonu (MLC) ile antifungal etki gösterirken, kantaron uçucu yağının MIC değeri 125 µl/ml ve MLC değeri 1000 µl/ml olarak bulunmuştur. Bulgular, özellikle zencefil uçucu yağının *S. ferax*'a karşı etkili bir doğal antifungal ajan olarak değerlendirilebileceğini göstermektedir.

Anahtar kelimeler: Antifungal aktivite, disk difüzyon, ITS bölgesi, tıbbi bitki, tüp dilüsyon.

INTRODUCTION

Saprolegniales is an order of oomycetes, and their life is completely dependent on water, hence, they are often called "water molds". This order has both pathogenic and saprophytic lifestyles. *Saprolegnia*, the largest genus of the family Saprolegniaceae, containing 25 species, has a wide distribution worldwide and shows pathogenic character in the embryonic and adult stages of fish and amphibians (Pavić et al., 2022). The organism is usually saprophytic, but it often becomes pathogenic when fish are stressed, injured, or have weakened immune systems. These pathogens cause a disease known as saprolegniasis, which is characterized by white or gray cotton-like hyphae on areas of the skin, gills, and fins of fish. If the infection is not treated, the hyphae invade the underlying tissues and blood vessels, leading to disrupted osmoregulation, respiratory failure, loss of balance, and ultimately death of the infected fish (Tedesco et al., 2019). The pathogens also infect unfertilized or dead eggs, aggregating them together due to hyphae and spreading the infection to other healthy eggs in the same environment. Saprolegniasis is one of the biggest problems for the aquaculture industry, especially in hatcheries for rainbow trout (*Oncorhynchus mykiss*) production.

Although various chemical substances such as formaldehyde, hydrogen peroxide, chloramine-T, batticon (polyvinylpyrrolidone iodine), apple vinegar, rock salt, and potassium permanganate have been used in different doses for disinfection against fungal infections during the incubation phase of rainbow trout eggs (Balta & Taşkın, 2022; Abdullahoğlu & Balta, 2023), malachite green has been reported to be particularly effective in controlling *Saprolegnia* infections. However, Saprolegniosis has re-emerged and become a serious problem in aquaculture after the ban of malachite green in the EU at the beginning of the 2000s due to its toxicity and carcinogenic effects (Elameen et al., 2021). There was an urgent necessity to create alternative strategies to address saprolegniosis due to the prohibition of malachite green (Sandoval-Sierra et al., 2014). Hence, numerous chemicals such as formalin, boric acid, peracetic acid, clotrimazole, copper sulfate, benzoic acid, hydrogen peroxide, and borneol have been tried to control *Saprolegnia* infections. Formalin is the most widely used among the other treatments, nowadays. The formalin treatment seems to be the only economically efficient way of controlling *Saprolegnia* infections in aquaculture. However, their use has been limited due to their negative environmental and fish health effects (Kumar et al., 2020). Therefore, the search for effective and safe natural therapeutic agents against *Saprolegnia* has increased. Medicinal plants have been reported to have antimicrobial (bacterial, fungal, viral, and ectoparasitic) effects thanks to their numerous bioactive components (Reverter et al., 2014). Specifically, medicinal plants antifungal activity proven against fungal diseases on rainbow trout (*O. mykiss*) eggs (Hoskonen et al., 2015; Metin et al., 2015; Ozdemir et al., 2022; Ozil et al., 2022), carp (*Cyprinus carpio*) (ALsafah & AL-Faragi, 2017), crayfish eggs (Koca & Cevikbas, 2015) and shrimp (*Litopenaeus vannamei*) juveniles (Mansour et al., 2023). In addition, the use of medicinal plants is becoming increasingly widespread due to their biodegradable properties, leaving no residue in living tissues (Dawood et al., 2021).

St. John's Wort (*Hypericum perforatum*) is a member of the genus *Hypericum*. This genus has 400 species worldwide, native to West Asia, North Africa, Madeira, the Azores, and Europe. It also occurs naturally in many other parts of the world, especially in North America and Australia. *H. perforatum* has many medicinal applications that aim to treat skin wounds, eczema, burns, digestive disorders, and psychological disorders. In addition, it has been reported that the plant shows strong antimicrobial activity against bacterial and fungal species with its hyperforin, hypericin, quercitrin, hyperoside, avicularin, rutin, quercetin, and kaempferol components (Saddiqe et al., 2010). *Zingiber officinale*, commonly known as ginger, belongs to the Zingiberaceae family, and it is an important medicinal herb widely used as a spice, flavoring agent, and herbal medicine worldwide. It is used in complementary medical practices to treat various diseases, such as nausea, vomiting, asthma, cough, inflammation, anorexia, constipation, and pain (Dhanik et al., 2017). Studies have reported that the plant has antifungal, antibacterial, and anthelminthic activity (Chen et al., 2008; Chang et al., 2012).

This study identified the molecular cause of fungal infection in rainbow trout eggs. It also investigated the antifungal activity of St. John's wort (*H. perforatum*) and ginger (*Z. officinale*) oils against the identified *Saprolegnia* sp.

MATERIAL AND METHOD

***Isolation of Saprolegnia strain*:** The strain of *Saprolegnia* was isolated from rainbow trout (*O. mykiss*) eggs collected from a commercial rainbow trout farm in Çameli district of Denizli province, following the method described by Diler and Timur (1995). Briefly, infected rainbow trout eggs were directly placed onto Glucose Yeast Extract Agar (GY agar – glucose: 1.0 g, yeast extract: 0.2 g, K_2HPO_4: 0.204 g, $Na_2HPO_4 \cdot 12H_2O$: 0.0596 g, agar: 2 g, deionized water: 100 ml). Penicillin-streptomycin (500 µm/ml) supplemented agar plates were incubated at 20°C for 3–5 days. After incubation, sterile hemp seeds (*Cannabis sativa*) were placed on the fungal culture to allow the fungal hyphae to colonize the hemp seeds. Subsequently, the fungus-colonized hemp seeds were transferred into sterile tubes containing distilled water and kept at 20°C for 48 hours to induce zoospore release from the zoosporangium. The

inoculum containing spores was then plated onto GY agar using a sterile pipette. After incubation at 20°C for 3–5 days, a 1 cm² section was cut from the GY agar using a sterile scalpel and transferred to a new culture medium for purification (Diler & Timur, 1995). The isolates were then examined macroscopically (colony morphology, shape, and color, growth pattern) and microscopically (presence of septate walls, sexual organ structure, spore size and arrangement) (Diler, 1992).

Molecular identification of Saprolegnia: Fungal DNA isolation was made using the commercial plant and fungi DNA isolation kit (EurX, Poland). Purity and amount of the isolated DNA samples were controlled using spectrophotometric measurement on a Nanodrop 2000 (Thermo Scientific, USA). In the PCR, the primers targeting ITS1-ITS4 regions of fungal DNA were used for conventional PCR. PCR reactions were performed with a commercially available Taq polymerase PCR kit (Solis Biodyne, Estonia). ITS1 -5' TCCGTAGGTGAACCTGCGG 3' and ITS4 -5' TCCTCCGCTTATTGATATGC 3' primers were used, and the PCR conditions given in Table 1 were used for conventional PCR. Thermal cycling was performed in a Biorad C-1000 according to Bahrioğlu et al. (2024) with minor changes. Briefly, the sequences were amplified with the following PCR conditions: 5 min denaturation step at 95 °C, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s, and 60 s of extension at 72 °C, and final extantion step at 72 °C for 5 min.

Table 1. The components, concentrations, and volumes used in the PCR.

Component	Stock Concentration	Volume (µl)
PCR Buffer	10X	2
$MgCl^{+2}$	25 mM	1.5
dNTP mix	20 mM	0.5
ITS1 Primer	10 µM	0.5
ITS4 Primer	10 µM	0.5
Taq DNA Polymerase	5U/µl	0.1
DNA template		3.0
DEPC-treated water		11.9

The amplicons obtained were visualized under UV light by electrophoresis on a 1% agarose (ethidium bromide) gel prepared (1x TAE buffer) at 100 volts for 90 minutes. The HighPrep™ PCR Clean-up System (MAGBIO Genomics, USA) purification kit was used for amplicon purification. An ABI 3730XL Sanger sequencer (Applied Biosystems, USA) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used for Sanger sequencing. Nucleotide reads obtained with the ITS1 and ITS4 primers were contig-formed. The CAP contig assembly algorithm in BioEdit software was used to generate a consensus sequence.

The determined sequence was submitted to NCBI GenBank (Accession Number: PV034298). The obtained sequence has also been submitted to the NCBI Blast Nucleotide Search to find similar sequences. Various sequences were randomly selected for further analysis. Sequences were representative of various *Saprolegnia* species. The multiple sequence alignment was conducted using the gene sequences from the search results. Multiple sequence alignments were performed with the ClustalW algorithm. MEGA X software was used to calculate BIC scores (Bayesian Information Criterion) for the maximum likelihood phylogenetic tree (Bahrioğlu et al., 2024). The Tamura-3 parameter model with gamma distribution (+G) had the smallest BIC value and was considered to describe the substitution pattern best. Therefore, the maximum likelihood (K92+G) model was used to build a phylogenetic tree of *Saprolegnia* species.

Preparation of plant oils and Gas Chromatography-Mass Spectroscopy (GC-MS) analysis: The ginger oil (*Z. officinale*) and St. John's wort oil (*H. perforatum*) were obtained from a commercial company (Manolya Doğal ve Aromatik Ürünler, Türkiye). The chemical content analysis of the oils was carried out by gas chromatography analysis at Süleyman Demirel University's Innovative Technologies Application and Research Center (YETEM). Gas chromatograph analysis was made in a Hewlett-Packard 6890 series (Perkin Elmer (PE) Auto System XL, USA) equipped with a flame ionization detector (FID). Gas chromatography was performed according to Özil et al. (2022).

Determination of in vitro antifungal effects of plant oils: Tube dilution and disk diffusion methods were used to determine the antifungal effects of ginger (*Z. officinale*) and St. John's wort (*H. perforatum*) oils.

Disk diffusion and tube dilution: The disk diffusion method was carried out to determine the fungistatic activity of plant oils inhibiting fungal growth. Fungal inoculum for the test was obtained from cultures grown on GY agar medium. Test concentrations (2.5-1000 µl/ml) were prepared by dissolving the oils in dimethyl sulfoxide (DMSO). A positive control (10 µl/ml formaldehyde) and a negative control (DMSO) were designated for further processing. Diffusion disks (6 mm) were impregnated with 25 µl of the prepared treatment groups. Fungi inoculated GY agar was then incubated for five days at 20°C. Each concentration was run in triplicate. The inhibition zones were measured as millimeters, and the value inhibiting 50% of the fungus was determined as the minimum fungistatic concentration (MIC) (Tampieri et al., 2003; Pirbalouti et al., 2009; Pirbalouti et al., 2010). The fungicidal activity was determined by the tube dilution method. Fungicidal activities were determined where the treatment concentration kills 99.9% of the fungal inoculum. To obtain fungal inocula, sterile hemp seeds were placed around the cultures on GY-PS agar, incubated for 3 to 5 days at 20ºC, and fungal hyphae colonized on hemp seeds. The oils were dissolved in DMSO, and 1000-2.5 µl/ml concentrations were prepared in sterile tubes. In addition, two different tubes were used as positive

control (formalin 10 µl/ml) and negative control (DMSO). The fungal inocula were added to these tubes and subjected to a seven-day incubation period at 20°C. Mycelium in the tubes was observationally evaluated, and the concentration that inhibited 99.9% of all the fungal inoculum was determined to be the minimum lethal concentration (MLC) (Pirbalouti et al., 2009).

Statistical analysis: The antifungal properties of ginger (*Z. officinale*) and St. John's wort (*H. perforatum*) oils were statistically analyzed using SPSS 19. The significance level of the parameters in the present study was presented as mean ± standard deviation (SD). Data were controlled for normality using the Shapiro-Wilk test. The results were analyzed using one-way ANOVA and Duncan's multiple comparison test. Statistical differences were accepted where the p-value was below 0.05.

RESULTS

Identification of Saprolegnia species: When the fungal colonies growing on GY agar were examined morphologically, long, white, and cottony structures were observed. When these colonies were examined microscopically (100x and 40x), they had branched, septa-less hyphae and large and dense mycelium. Amplification of the ITS region from the genomic DNA of the isolates using ITS1 primers produced amplicons of approximately 1326 bp. The nucleotide sequences obtained from Sanger sequencing were searched in NCBI BLAST. According to the results of the nucleotide search, the ITS sequence obtained in the study showed a 100% match with different *S. ferax* strains. The ITS region of 15 strains of *S. australis*, *S. parasitica*, *S. ferax*, and *S. diclina* species registered in the NCBI GenBank database were used for phylogenetic analysis.

The phylogenetic tree (Figure 1), constructed using the Maximum Likelihood method with the Tamura-3 parameter model, comprises 16 nucleotide sequences and 668 positions in the final dataset. The model selection criteria indicate that the Tamura 3-parameter model with gamma distribution had a Bayesian Information Criterion (BIC) score of -1522.77. Initial trees were generated by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances. Pairwise distances were estimated using the Tamura 3-parameter model. A discrete Gamma distribution was used to model the evolutionary rate differences among sites (5 categories, parameter = 0.1578). The estimated transition rate (R) of 1.63 indicates moderate nucleotide variation. The phylogenetic tree contains several well-supported clades, as the bootstrap values indicate. In particular, the bootstrap value of 100 between *S. ferax*, *S. parasitica*, *S. australis*, and *S. diclina* reflects a high accuracy of molecular identification.

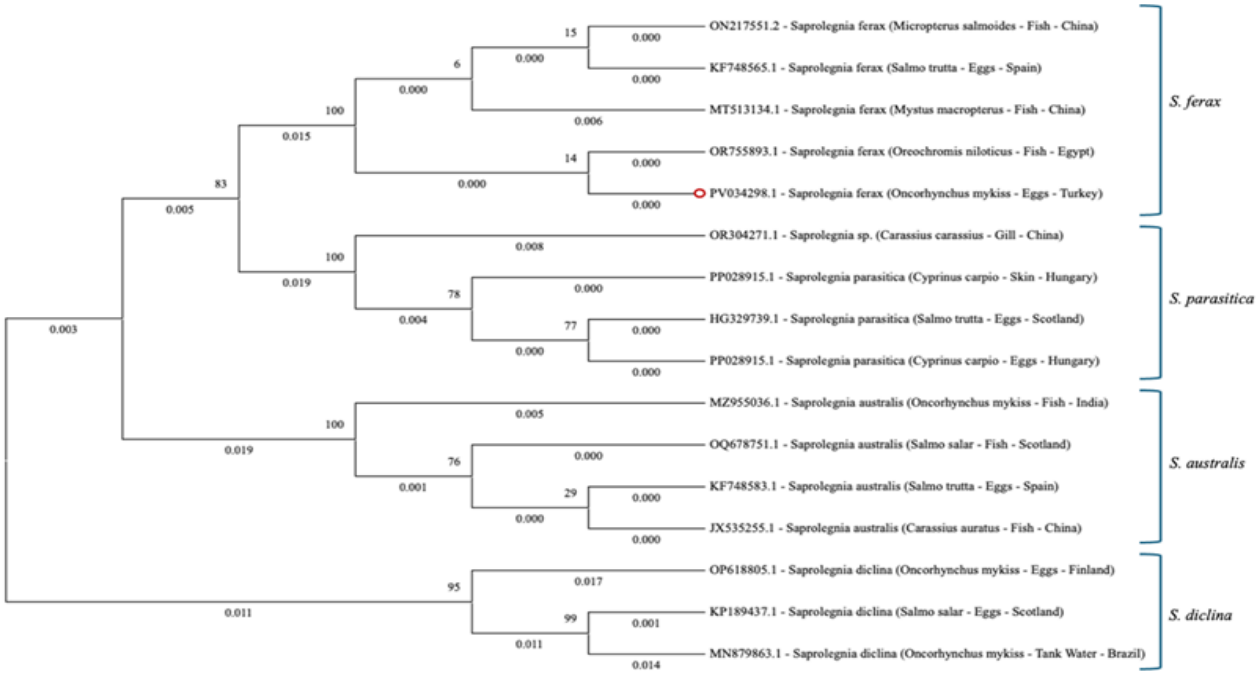

Figure 1. The phylogenetic tree of the ITS region of *Saprolegnia* species derived from the NCBI GenBank database. The red-circled branch is the *S. ferax* strain used for the present study.

GC-MS analysis results of plant oils: According to the chemical content analysis of plant oils, the main components were determined to be α-curcumene (22.4%), zingiberene (12.2%), farnesene (7.7%), (+)-β-cedrene (6.5%), 1,8-cineole (5.4%), camphene (5.1%), citral (4.7%) in ginger (*Z. officinale*), and α-pinene (27%), β-pinene (10.3%), β-caryophyllene (6.7%), germacrene-d (6.3%), α-selinene (4.5%) in St. John's wort (*H. perforatum*).

In vitro antifungal activity: The disk diffusion and tube dilution tests showed that the ginger (*Z. officinale*) and the St. John's wort (*H. perforatum*) oils had antifungal effects against the tested *S. ferax* strain (Figure 2). It was determined that ginger (*Z. officinale*) oil was effective in the concentration range of 15.62-1000 µl/ml. This oil was found to be more effective on the fungal strain compared to the positive control group (26.50 ± 0.70^{b} mm) with zone diameters of 31.00 ± 1.41^{a} mm and 28.50 ± 2.12^{ab} mm at concentrations of 1000 and 500 µl/ml ($p<0.05$) (Figure 2 and 3). St. John's wort (*H. perforatum*) oil showed an antifungal effect in the 125-1000 µl/ml concentration range, but this effect was lower than the positive control group (Figure 2).

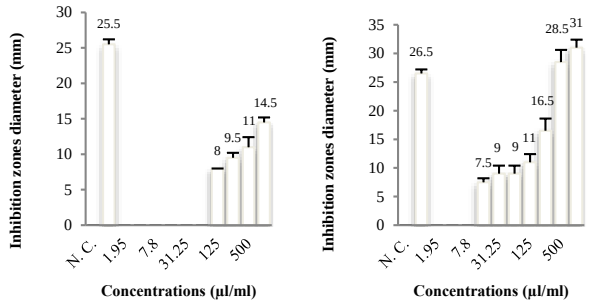

Figure 2. Inhibition zone diameter values of St. John's Wort (*H. perforatum*) (left) and ginger (*Z. officinale*) plant oil (right) obtained from the disk diffusion test result.

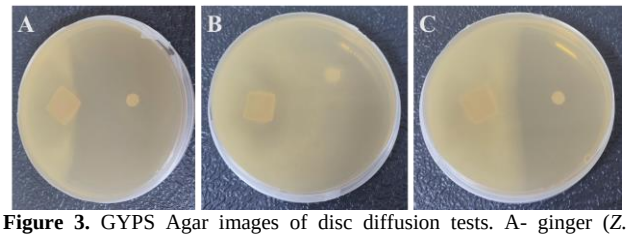

Figure 3. GYPS Agar images of disc diffusion tests. A- ginger (*Z. officinale*) plant oil. B- Negative control. C- St. John's Wort (*H. perforatum*).

The disk diffusion tests showed that the MIC values of the *S. ferax* isolate were 15.62 µl/ml for ginger (*Z. officinale*) oil and 125 µl/ml for St. John's Wort (*H. perforatum*) oil. According to the tube dilution test, the ginger (*Z. officinale*) oil killed the fungi at 250 µl/ml, and St. John's Wort (*H. perforatum*) oil was determined to have a fungicidal effect at 1000 µl/ml, and this concentration was recorded as MLC.

DISCUSSION AND CONCLUSION

Saprolegnia ferax has been reported to be a pathogenic fungal species commonly found in fish (Sandoval-Sierra et al., 2014) and amphibians (Romansic et al., 2009) in freshwater environments worldwide. Since its first description by Kützing (1843), it has been reported from various locations including America, Asia, Australia, and Europe (Johnson et al., 2002). Morphological and physiological characteristics of *S. ferax* species in Atlantic salmon (*Salmo salar* L.) (Stueland et al., 2005), Chum salmon (*Oncorhynchus keta*) eggs (Sakaguchi et al., 2019), yellow catfish (*Pelteobagrus fulvidraco*) eggs (Cao et al., 2012), crayfish (*Astacus astacus*) (Kozubikova-Balcarova et al., 2013), Argentinian silverside (*Odonthestes bonariensis*) juveniles and eggs (Pacheco Marino et al., 2009), *Odontesthes bonariensis* fish (Pacheco-Marino et al., 2011), Nile tilapia (*Oreochromis niloticus*) fish (El Gamal et al., 2023), from water, fish and eggs in a rainbow trout (*O. mykiss*) farm in Mexico (Vega-Ramírez et al., 2013), from Atlantic salmon (*Salmo salar*), rainbow trout (*O. mykiss*) and king salmon (*O. tshawytscha*) in Chile (Sandoval-Sierra et al, 2014), *Cyprinus carpio*, *Carassius carassius* and *Perca fluviatilis* (Markovskaja et al., 2024), and water, fish and egg samples from a trout farm in Croatia (Pavíc et al., 2022).

S. ferax was first isolated as a pathogenic agent from the eggs and juveniles of rainbow trout raised in aquaculture facilities in the 1990s in Turkey (Diler, 1992). Identification of *S. ferax* was done using the morphological methods in the first report. Nowadays, disease monitoring and accurate identification of pathogens have become important aspects in the fight against pathogens. The increasing use of molecular methods allowed us to combine morphological methods with molecular methods in diagnosis, and this application plays an effective role, especially in the identification of morphologically similar *Saprolegnia* species (Tedesco et al., 2021). In this study, the causative agent of the disease was identified as *S. ferax* fungal species, as a result of inoculations and molecular examinations from infected rainbow trout eggs.

In particular, it has been reported that the hyphae of *S. ferax* species reach deeper parts of the egg chorion compared to other fungal species, and therefore, its pathogenicity is higher (Hardy et al., 2023). In this respect, it is important to isolate the presence of this species, which has high pathogenicity in eggs, from rainbow trout aquaculture facilities and to investigate the treatment methods. In this direction, antifungal activity of different medicinal plants against various pathogenic fungi has been investigated in many studies (Gormez & Diler, 2014; Metin et al., 2015; ALsafah & AL-Faragi, 2017; Emara et al., 2020). When the literature is examined, it is stated that the antifungal effects of plants are related to their phytochemicals. It has been reported that the antifungal activity of these phytochemicals is linked to various mechanisms such as disruption of the cell membrane, inhibition of cell division, hyphae development, and metabolic pathways (Bouyahya et al., 2020).

Different extraction methods are used to obtain phytochemicals from medicinal plants. The most common

of these methods are extracts and essential oils. Although plant extracts and essential oils are of plant origin, they show significant differences in terms of content, extraction method, and areas of use (Bolouri et al., 2022). Extracts are obtained from parts of the plant such as leaves, roots, and flowers with the help of water, ethanol, or other solvents and contain a wide range of metabolites, especially polyphenolic compounds such as flavonoids, saponins, tannins, and polysaccharides (Cao et al., 2013). In contrast, essential oils consist of low molecular weight, volatile, and aromatic compounds synthesized as a result of the secondary metabolism of the plant and are generally obtained by methods such as steam distillation (Mohamed & Alotaibi, 2023). The main components of essential oils are terpenes and phenylpropanoids, and the content and ratios of these compounds vary depending on the plant species, the organ used, and the harvest and processing conditions (Bolouri et al., 2022; Mohamed & Alotaibi, 2023).

In this study, the high phenolic compound contents of ginger (*Z. officinale*) and St. John's wort (*H. perforatum*) essential oils, as determined in this study, and their antifungal activity on *S. ferax* show significant results when compared to previous studies. Ginger oil (*Z. officinale*) presented a strong antifungal effect with a MIC value of 15.62 µl/ml and a MLC value of 250 µl/ml, while this effect was lower for St. John's wort (*H. perforatum*) oil with a MIC value of 125 µl/ml and a MLC value of 1000 µl/ml.

Mostafa et al. (2020) showed that among the plant extracts of *Punica granatum*, *Thymus vulgaris*, *Nigella sativa*, and *Zingiber officinalis* applied against *Saprolegnia diclina* isolated from Nile tilapia, only the ethanol extracts of *P. granatum* and *T. vulgaris* inhibited mycelial growth with MIC of 200 and 400 ppm, respectively. This finding suggests that antifungal activity may vary depending on the active compound profiles and concentrations of plant extracts. Similarly, the study by Caruana et al. (2012) demonstrated that the plant extracts of *Atractylodes macrocephala*, *Zingiber officinale*, *Chrysanthemum* spp., and *Yucca* spp. exhibited antifungal efficacy at a dose of 10 ppm against *Saprolegnia australis* isolated from infected brown trout (*Salmo trutta* L.), supporting the notion that antifungal activity can be observed even at low doses. In the study by Cao et al. (2013), *Zingiber officinale* extract was found effective with an MIC value of 8192 mg/l against the oomycete water mold *Achlya klebsiana* isolated from yellow catfish (*Peleobagrus fulvidraco*) eggs, indicating that formulation differences between extracts and essential oils may play a decisive role in antifungal efficacy. Furthermore, the study by Xue-Gang et al. (2013) evaluating *Zingiber officinale* extract in the concentration range of 15.6–500 mg/l against *Saprolegnia* sp. stock strain did not observe significant efficacy, highlighting that the preparation method of the extract and the concentration of active compounds are critical factors in determining antifungal activity.

Although the varying levels of antifungal activity observed in the literature have been associated with different extract forms and phytochemical compositions, the fact that the same plant (*Z. officinale*) exhibits different MIC and MLC values against different *Saprolegnia* species suggests that this plant is not suitable for treating all fungal species and has a species-specific effect. For *S. ferax* isolated from the skin of grass carp (*Ctenopharyngodon idella*), the medicinal plant extracts obtained from *Syzygium aromaticum*, *Magnolia officinalis*, *Melaphis chinensis*, *Euphorbia fischeriana* Steud, and *Sophora flavescentis* were found effective with MIC of 500, 62.5, 250, 62.5, and 250 mg/l, respectively (Huang et al., 2015). This finding indicates that antifungal efficacy is closely related to the plant's phenolic content intensity and active compound composition. Similarly, Hussein et al. (2000) reported MIC of 500, 250, and 125 µg/ml and fungicidal concentrations of 1,000, 500, and 250 µg/ml for *Saprolegnia parasitica*, *S. diclina*, *S. ferax*, *S. salmonis*, *Achlya klebsiana*, and *Aphanomyces piscicida* using a 10% v/v eugenol solution in DMSO. Additionally, MIC of 250, 125, 250, and 63 µg/ml were obtained with the eugenol-based FA 100 solution, emphasizing the critical role of formulation in evaluating antifungal activity.

In the study by Ackah et al. (2025) on a reference strain of *S. ferax* isolated during past Saprolegniasis outbreaks in Nile tilapia (*Oreochromis niloticus*), *Azadirachta indica* (neem leaf), *Vernonia amygdalina* (bitter leaf), and *Terminalia catappa* (Indian almond leaf) extracts, along with potassium permanganate ($KMnO_4$), were tested. The complete inhibition of mycelial growth with $KMnO_4$ and *T. catappa* extract at an MIC of 5 mg/mL demonstrates that natural products can exhibit strong antifungal activity even at low concentrations. Furthermore, Macchioni et al. (1999) investigated the water, methanol, and water+ethanol extracts of *Artemisia verlotorum* and *Santolina etrusca* against *Saprolegnia ferax* and found that *A. verlotorum*'s water extract had an MIC of 1%, while its methanol and water+ethanol extracts exhibited stronger efficacy with an MIC of 0.25%, highlighting the significant impact of solvent choice and extraction methods in assessing antifungal activity. Overall, these studies suggest that the antifungal efficacy of different plant extracts and essential oils varies significantly depending on multiple factors, including the fungal species tested, application method, solvent used, extraction techniques, and active compound concentrations. In this context, the MIC value of 15.62 µl/ml obtained for ginger oil in our study demonstrates a

notably strong antifungal effect compared to previous literature findings, particularly when considering the differences in active compound profiles and concentration between extracts and essential oils.

In conclusion, the use of plant-derived products as antifungal agents offers environmentally friendly and effective alternatives against fungal pathogens in aquatic environments. Future studies focusing on the isolation of active compounds and the detailed elucidation of their mechanisms of action are of great importance. Additionally, when utilizing a plant species for its antifungal properties, its species-specific effects should not be overlooked, and molecular identification of the target pathogen is recommended for optimal application.

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