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ANTIPARASITIC EFFECTS OF *Artemisia absinthium* L. IN THE TREATMENT OF HEXAMITIOSIS IN RAINBOW TROUT (*Oncorhynchus mykiss*) CULTURE

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Abstract: In recent years, the inefficacy and adverse effects of synthetic drugs and therapeutic agents used against the increasing prevalence of diseases in aquaculture have highlighted the need for introducing medicinal plants with antimicrobial properties into the sector. The use of plant-based products in the treatment of cultured fish species holds significant potential, particularly in supporting organic aquaculture practices. In this study, the antiparasitic activity of the ethanol extract of *Artemisia absinthium* L. (wormwood) was investigated against Hexamitiosis disease caused by *Spironucleus salmonis* in juvenile rainbow trout. The plant extract was incorporated into fish feed at doses of 1.5, 2.0, and 2.5 g/kg and administered to infected fish with an average body weight of 1.5–2.0 g for both short-term (7 days) and long-term (21 days) treatment periods. The results revealed that the *A. absinthium* extract exhibited antiparasitic efficacy against *S. salmonis* on both the 7th and 21st days. Cumulative mortality rates decreased in all experimental and positive control groups, with the 21-day application proving more effective than the 7-day treatment. The highest antiprotozoal activity was observed with the 2.5 g/kg dose on the 21st day, showing a statistically similar effect to that of the positive control ($p<0.05$). Based on these findings, it was concluded that ethanol extracts of *A. absinthium* could be used as a natural and alternative antiparasitic agent for controlling *S. salmonis* infections in rainbow trout aquaculture.

Key words: *Artemisia absinthium* L., *Spironucleus salmonis*, rainbow trout (*Oncorhynchus mykiss*), antiparasitic, treatment

Introduction

Spironucleus is a flagellated single-celled parasite that can be found in the intestines of fish. *Spironucleus* infections are becoming serious, especially under high-density fish farming conditions. *Spironucleus* has been found in many other fish species, including salmonids and tilapia and aquarium fish. Infections primarily affect the intestine and tend to be chronic in nature. It has been reported that the disease is transmitted by oral ingestion of cysts and trophozoites of the parasite. Fish population density is effective in the occurrence of the disease (Woo, 1999). The disease plays an important role in cichlid cultivation and is frequently the most important profit-limiting factor in intensive aquaculture. Dimetridazole and metronidazole are commonly administered through feed in the treatment of *Spironucleus* infection. However, due to the adverse effects of metronidazole on DNA and cell structure, its use in aquaculture within European Union countries is prohibited by Directive No. 613/98. Thus, there is an urgent need to systematically evaluate the traditionally used medicinal plants for getting an alternative measure for controlling Spironucleosis in aquaculture (Noor El Deen and Mohamed, 2009; Militz et al., 2013).

The use of phytochemicals as antiprasitic drugs for fish health may help to support sustainable and environmentally acceptable treatment applications (Park et al., 2011; Hao et al., 2012). There are previous studies reviewing investigations that have examined the antiparasitic effects of plants on fish parasites (Wink, 2012; Ramudu and Dash, 2013; Reverter et al., 2014; Syahidah et al., 2015; Lieke et al., 2019). In recent years, many researchers have focused on the use of herbal plants against parasitic species in aquaculture (Chitmanat et al., 2005; Puk and Guz, 2014; Williams et al., 2016; Trasvina-Moreno et al., 2017; Yildiz et al., 2019).

Traditionally, *Artemisia absinthium* L., a plant widely distributed and naturally occurring in Anatolia, particularly in the Mediterranean region, exhibits diverse biological properties such as antihelminthic, antibacterial, antifungal, antioxidant, antimalarial effects, with terpenoids and flavonoids as active ingredients (Kordali et al., 2005; Bora and Sharma, 2010). *A. absinthium* has always been of pharmaceutical and botanical importance and used to manage several disorders including hepatocyte enlargement, hepatitis, gastritis, jaundice, wound healing, splenomegaly, dyspepsia, indigestion, flatulence, gastric pain, anemia, and anorexia.

The risks of chemical use for eliminating the parasites include anthelmintic resistance, risk of residue, environmental contamination, and toxicity to hosts. Therefore, this study aims to determine the potential *in vivo* antiparasitic activity of *A. absinthium* L. extract as an alternative for controlling Spironucleosis (Hexamitiasis) in rainbow trout.

Materials and Methods

Infected juvenile rainbow trout samples were obtained from trout farms in the Aksu-Isparta region in January-February. The intestinal tissues of infected fish were examined under a microscope. Spironucleus infestation is easily confirmed by making a fresh-tissue squash preparation of the intestine and examin-ing it with a light microscope at 200 and 400x magnification. The flagellates have a distinctive, ovoid to pyriform ("pear") shape and rapid, erratic movement. They are most easily seen in the mid to posterior intestine. The infected rainbow trout were transported to Fisheries Faculty Research and Application facility. The experiment was set up for 50 fish each group with an average weight of 1.5-2 g, as triplicate. Fish were placed in 400-liter round fiberglass tanks. The research was carried out over two different periods: short-term (7 days) and long-term (21 days) and a running water system was used.

Preparation of ethanol extract and essential oil of *A. absinthium* L.

A. absinthium L. was defined by Prof. Dr. Hasan ÖZÇELİK from Süleyman Demirel University, Faculty of Sciences, Department of Biology. The chemical structure of *A. absinthium* L. was determined by the Gas chromatography device (using GC/MS apparatus) at the Experimental and Observational Research Laboratory of Süleyman Demirel University. For preparation of plant extract, after keeping 15 g of dry plant sample in 50 ml of ethanol for 2 hours, it was passed through Whatman No1 filter paper for filtration, evaporation was carried out with the help of a water bath, and the resulting extract was stored at -18°C until use. To obtain essential oil, the crude powder samples (200 g) were subjected to hydrodistillation for 3 hours using a Clevenger-type apparatus.

Experimental diets

Different concentrations of ethanol extract (1.5, 2.0, 2.5 g/kg) were added to the mixture together with sunflower oil (0.05 mL/kg). Juvenile rainbow trout were fed at 3% of their body weight for 7 days (short term) and 21 days (long term). The negative control group was fed with commercial trout feed at 1% of their body weight. In the positive control group, they were fed trout feed supplemented with 0.5 mg/kg of Metronidazole. All procedures on experimental fish were carried out in accordance with national ethical rules approved by the Süleyman Demirel University Animal Care and Use Committee (Republic of Turkey Süleyman Demirel University Experimental Animal Use Ethics Committee, E-77211729-804.01-18968).

Determination of the therapeutic effect of *A. absinthium* L.

Determination of survival rate

The survival percentage of the fish (RPS = Relative Percent Survival); it was calculated according to the formula (Amend, 1981):

RPS = [1 - (Mortality in the *A. absinthium* L. fed group (%) / Mortality in the control group (%))]

Determination of the number of parasites in tissues

Parasite density was determined by counting the parasites in the intestine and feces according to Tojo and Santamarina (1998). For this purpose, after the fish were stunned with phenoxyethanol (0.1-0.5 ml/L), slide coverslip preparations prepared from feces and intestinal contents were examined under a microscope at 400x magnification. In the evaluation; if more than 50 parasites are found in the microscopic area, 4 points (high (+++)), 3 points (medium (++) between 10-50), 2 points (low (+) between 1-10), 1 point (if there was 1 piece, it was interpreted as minimal (+/-)), if no interference was found, it was interpreted as zero score (-).

Data Analysis

The data obtained in the experiment were evaluated with the ANOVA test (Duncan multiple comparison test) in the SPSS 16.0 package program (SPSS Inc, Chicago, IL, USA). When comparing the importance levels of various parameters examined in the experiment, the results are given as mean value and standard deviation. The significance level was chosen as $p<0.05$.

Results

***A. absinthium* L. GC-MS analysis**

The composition of *A. absinthium* L. was analyzed by GC-MS and the major components were sabinol (42.22%), chrysanthenylacetate (14.73%), epoxy-ocimene (4.97%), thujone (2.19%), d-isothujone (3.97%).

Result of relative survival rate (RPS) and cumulative mortality

According to research findings, it was determined that cumulative mortality (%) decreased and survival rates increased in fish fed with *A. absinthium* L. extract, similar to the positive control, for 21th day (Table 1).

Table 1. Effect of *A. absinthium* L. extract on relative survival rate (RPS) and cumulative mortality in infected rainbow trout

	Cumulative Mortality (%)		RPS (%)	
	7th day	21th day	7th day	21th day
1.5 g/kg	8.67±0.67^{ab}	14.00± 0.67^{bc}	12.50±1.25^{a}	29.91±1.34^{a}
2.0 g/kg	8.67±2.00^{ab}	10.00±2.00^{ab}	25.90±1.16^{a}	49.11±1.34^{ab}
2.5 g/kg	8.00±0.00^{ab}	4.00±1.00^{a}	19.65±5.35^{a}	78.57±2.14^{b}
Positive control	9.34±1.34^{ab}	14.00±2.00^{bc}	19.65± 5.35^{a}	30.36±5.33^{a}
Negative control	10.00±0.67^{ab}	20.00±1.33^{c}	-	-

*Different letters in the same column are statistically significant ($p<0.05$)

Effect of *A. absinthium* L. extract on the number of parasites in the digestive canal of rainbow trout

It was determined that *A. absinthium* L. extract was not effective on the 7th day, but on the 21st day, the group treated with 2.5 g/kg of *A. absinthium* L. was found to be more effective in terms of the intensity and prevalence of infection than positive control and other groups ($p<0.05$) (Table 2).

Table 2. Effect of *A. absinthium* L. extract on infection intensity and infection rate (prevalence) in infected rainbow trout

	Intensity of infection		Prevalence (%)	
	7th day	21th day	7th day	21th day
1.5 g/kg	++	+	83.21±1.06^{b}	54.26±1.40^{c}
2.0 g/kg	++	+	83.21±2.03^{b}	29.62±1.46^{d}
2.5 g/kg	+	-	86.95±0.12^{a}	3.47±0.04^{f}
Positive control	++	-	55.14±1.48^{d}	1.55±0.08^{f}
Negative control	+++	+++	88.14±0.79^{a}	100.00±0.00^{a}

*Different letters in the same column are statistically significant ($p<0.05$)

Discussion

The flagellated protozoan from the genus Spironucleus (Kolisko et al., 2005) causes significant losses in both food and ornamental fish production (Sterud et al., 1998; Paull and Matthews, 2001; Jørgensen and Sterud, 2006). They often cause disease when the host has low resistance or is adversely affected by

predisposing factors such as low oxygen content or overcrowding (Lom and Dykova, 1992). Metronidazole (l-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is a 5-nitroimidazole; a heterocyclic compound with a nitro group on the fifth position of an imidazole ring. It is the active compound of nitroimidazole, used in the treatment of infections induced by anaerobic bacteria and protozoa (Guz and Szczepaniak, 2009; Sangmaneedet and Smith, 1999). But has been banned from use in the treatment of food fish in Europe and the USA due to its potential carcinogenic properties, persistence in the environment and toxicity to aquatic organisms (Lanzky and Halling-Sorensen, 1997; Payne et al., 1999; Treves-Brown, 1999). The severe restrictions on the use of metronidazole highlight the need for alternative Spironucleus treatments in food and ornamental fish (Millet et al., 2011).

Medicinal plants have been shown to possess antiprotozoal activity, and their use in traditional medicine has led to the development of new antiparasitic drugs. Unlike conventional chemical treatments, medicinal plants are generally considered safe for the environment and do not pose a risk to human health. Additionally, many medicinal plants are readily available and inexpensive, making them an attractive option for aquaculture producers (Özil, 2023).

Antiprotozoal activity of medicinal plants is attributed to their chemical composition, such as monoterpenes (1,8-cineole, alfa pinene, camphor, linalool, sabinene, and thujone), sesquiterpenes (e-nerolidol, germacrene B, and cadalene), isoprenoides, and terpenoids that include aldehydes, ethers, alcohols (carvacrol, thymol, eugenol, and borneol), and sulfur- or nitrogen-containing compounds (diallyl disulfide and indole) (Morsy, 2017).

A. absinthium and its extracts have antiprotozoal activities. The extracts of the plant exhibited antiprotozoal action on several apicomplexan parasites (e.g., *Eimeria*, *Plasmodium*, *Toxoplasma*, *Babesia*, and *Theileria*) and other protozoan parasites (e.g., *Trypanosoma cruzi*, *T. brucei*, and *Leishmania infantum*) (Fernández-Calienes Valdés et al., 2008; Nozari et al., 2016; Batiha et al., 2019). Coccidiosis is one of the most important infections in livestock caused by *Eimeria* species and of greater economic importance due to high morbidity rates. Anticoccidial activity of *A. absinthium* extracts has been reported in ruminants as well as in poultry; however, the activity depends on the number of oocysts and the type of *Eimeria*. Aqueous *A. absinthium* extract at a dose of 3 mg/kg of feed per day induced a substantial reduction in the number of oocysts in broiler infected with *Eimeria tenella* and can be used as prophylactic treatment for moderate coccidiosis (Kostadinovic et al., 2012; Habibi et al., 2016).

In a study, *in vitro* the antiprotozoal properties of aqueous and alcoholic fractions *of A. hippocastanum, A. sativum, A. rusticana, O. vulgare and T. vulgare* was examined against *Spironucleus vortens* and concluded that extracts of these

plants are suitable candidates for antiprotozoal drug discovery for control of spironucleosis (Puk and Guz, 2014).

In a another study was to determine the *in vivo* antiparasitic activity of the *Artemisia campestris* (L) ethanol extracts for control of Spironucleosis (Hexamitiasis) in rainbow trout (*Oncorhynchus mykiss*). Treated rainbow trout with an average weight of 1.5-2.0 g, were fed with 7 and 21-day periods with the addition of plant extracts of 1.0, 1.5, 2.0, 2.5 and 3.0 g/kg. The composition of ethanol extract of *A. campestris* (L) was analyzed by GC-MS and the major components were acenaphthylene 1,2–dihydro (62.70%), n-octadecane (4.66%), capillin (4.95%), curcumene (4.45%), 2.4-pentadiynylbenzene (7.28%), benzaldehyde (2.53%), methacrolein (1.35%). Their results of study showed that cumulative mortalities were decreased in all treatment groups and positive control, compared to the negative control (Diler et al., 2018).

In this study, 58 different components were obtained in the chemical composition of the extract of *A. absinthium* and they are rich in monoterpene components which, sabinol (42.22%), thujone (2.19%), d- isothujone (3.97%). Terpenes interacts with the lipids in the cell wall, increase the permeability of the membrane. Naturally, disruption of the physicochemical structure causes in proton movement and electron flow and therefore transport in the cell and coagulation of the cell content (Erdoğan and Everest, 2013). In this research, *A. absinthium* was effective on *Spironucleus*. It has been understood that *Artemisia* species are an alternative for the protection of the aquatic ecosystem and sustainable aquaculture production and can contribute to organic production. It has been determined that higher doses are more effective than lower doses. It has been observed that *Artemisia* group herbal products and has no harmful effects on fish. In our research, the effective doses of the ethanol extract of *A. absinthium* L., was found for the treatment of fish pathogenic *Spironucleus* infections and it was determined that the long period (21 days) was more effective than the short period (7 days) in the treatment against the parasite ($p<0.05$).

Conclusion

In future studies, should be conducted on sectoral field practices for the treatment of *Spironucleus* infections and disease prevention strategies should be developed by giving importance to prophylactic studies.

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Conflict of interest
The authors declare that they have no conflict of interest.

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