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Evaluation of the anti-*Acanthamoeba* potential of *Myriophyllum spicatum* on *Acanthamoeba castellanii* trophozoites

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ABSTRACT

Myriophyllum spicatum is a submerged aquatic macrophyte known for its allelopathic and antimicrobial properties. This study investigated the amoebicidal activity of ethanolic *M. spicatum* leaf extract against *Acanthamoeba castellanii* trophozoites. Plant samples were collected from the Terme River (Samsun), extracted with ethanol, and tested at concentrations ranging from 1.5 to 48 µg/mL over 24, 48, and 72 hours. Trophozoite viability was assessed using trypan blue staining, and statistical analysis was performed with SPSS and Jamovi software. Results showed significant dose- and time-dependent reductions in trophozoite viability, with an IC50 value of 26.5 µg/mL at 72 hours. The highest inhibition was observed at 48 µg/mL, resulting in a 33% reduction in viability. Principal Component Analysis revealed distinct clustering of higher concentrations from lower doses and control. The study highlights *M. spicatum*'s potential as a natural anti-amoebic agent. Given the parasite's resistance to conventional drugs and the increasing threat of *Acanthamoeba* infections, particularly in developing regions, plant-based compounds offer promising alternatives. Further research is recommended to evaluate the extract's effects on the cyst stage and explore its therapeutic applications. This study is the first to report the anti-Acanthamoeba effect of *M. spicatum*, contributing novel insight into aquatic plant-based antiparasitic agents.

Keywords: Eurasian water milfoil, *Myriophyllum spicatum, Acanthamoeba castellanii, Amoebicidal activity*

Introduction

Myriophyllum spicatum (Haloragaceae) is a cosmopolitan aquatic plant and one of the most species-rich genera among aquatic angiosperms. Approximately 68 species of this genus have been documented worldwide. In Türkiye, two species of this genus are known: *Myriophyllum spicatum* and *M. verticillatum*. Some species are considered invasive macrophytes because of their rapid vegetative growth and strong competitive ability (Taş et al., 2018; Liao et al., 2020). *M. spicatum* is the most common and heavily managed invasive aquatic plant due to concerns about its impact on native plant communities and human recreation (Hoff & Thum, 2022). In Türkiye, *M. spicatum* (Eurasian watermilfoil) is widely found in lentic and lotic shallow water ecosystems (rivers, lakes, wetlands, irrigation systems), and it is a cosmopolitan submerged macrophyte. Macrophytes play important roles in aquatic ecosystems, including absorbing nutrients and heavy metals, reducing suspended solid loads, providing clean and clear water environments, improving water quality, and creating habitats for aquatic animals (Taş & Topaldemir, 2021; Ustaoğlu et al., 2022). Additionally, macrophytes are also studied in ethnobotany (Topaldemir & Taş, 2024).

Submerged macrophytes not only have effective nutrient uptake capabilities but also possess unique allelopathic properties. Some *Myriophyllum* species (*M. aquaticum* (Vell.) Verdc., *M. spicatum*, *M. verticillatum*) release allelochemicals (Gross & Garric, 2019). Allelochemicals such as tellimagrandin II and gallic acid, isolated from *Myriophyllum* extracts, have been demonstrated to affect phytoplankton growth negatively (Leu et al., 2002), and *M. spicatum* is known to have a strong ability to suppress the growth of the globally known cyanobacterium *Microcystis aeruginosa*, which forms harmful blooms (He et al., 2016; Jeong et al., 2021; Jeong et al., 2024). Allelochemicals, which contain phenolic compounds, leach from plants into the water column, causing either synergistic or antagonistic effects on other organisms (Gross & Garric, 2019; Wang & Liu, 2023). However, only a small portion of the metabolites present in the plants are released into the environment (Gross & Bakker, 2012).

It has been reported that *M. spicatum* can significantly impact the formation and distribution of mysids and fish larvae in coastal ecosystems, resulting in substantial mortality among mysids (Lindén & Lehtiniemi, 2005). *M. aquaticum* also produces chemicals with allelopathic effects, which impact zooplankton. For example, depending on the source of the total phenols used, significant differences in survival and reproduction were observed in cultures of the rotifer Brachionus havanaensis Rousselet. Rotifers exposed to total phenols extracted from the plant at concentrations of 12.2 μg/g did not survive for more than a week (Viveros-Legorreta et al., 2022).

The amount of phenolic compounds in aquatic plants varies depending on the region and season in which the plants are collected. A study examining the in vitro antimicrobial effects of *M. spicatum* collected from different aquatic systems in Türkiye (Miliç River, Samsun, and Ulugöl Lake, Ordu) reported that the ethanolic extracts of the plant exhibited effects on test organisms. The antimicrobial effect of samples from the Miliç River was found to be more effective than that of samples from Ulugöl Lake (Ertürk et al., 2020). The literature review revealed no studies investigating the anti-*Acanthamoeba* properties of *M. spicatum*.

A. castellanii, a free-living amoeba species commonly found in water and soil, can cause severe human infections and exists in both trophozoite and cyst forms. Belonging to the Acanthamoebidae family, this species proliferates through mitosis, allowing it to spread widely (De Lacerda & Lira, 2021).

A. castellanii is a resilient organism that thrives in various environments. It can be found in swimming pools, drinking and utility water systems, sewage, hot springs, aquariums, ventilation systems, healthcare facilities, dental treatment areas, dialysis units, contact lens storage cases, disinfectants, cell cultures, particular plant species, and among vertebrates. Humans can become infected through several routes, including the nasal passages, throat, infected brain or lung tissues, skin wounds, and corneal tissue, particularly in patients with keratitis (Aykur & Dagci, 2023; Kaynak et al., 2024).

A. castellanii can enter the human body through the respiratory system, eyes, and skin, leading to a variety of severe conditions. One of the most common is *Acanthamoeba* keratitis (AK), which is frequently seen in contact lens users and can cause painful eye infections, potentially leading to blindness if left untreated. Less common but potentially fatal diseases caused by this amoeba include Granulomatous Amoebic Encephalitis (GAE) and Cutaneous Acanthamoebiasis (Ceniklioglu & Duzlu, 2022).

The drugs used to treat *Acanthamoeba* infections are generally ineffective, and long-term treatments are often poorly tolerated by patients. Additionally, the drugs have serious side effects, and the parasite's resistance to the medications complicates the treatment process. Furthermore, the cyst form of the parasite is resistant to both drugs and disinfectants, and its eukaryotic structure makes treatment more challenging. For these reasons, ongoing research aims to develop effective, well-tolerated, and non-cytotoxic drugs for both the trophozoite and cyst forms of *Acanthamoeba* (Fiori et al., 2006; Kaynak et al., 2019).

In this study, the effects of the ethanolic extract of *M. spicatum* collected from the Terme River (Samsun) on the cell viability of *Acanthamoeba castellanii* trophozoites were investigated. The effects of different concentrations of *M. spicatum* extract at various time intervals were determined, and the amoebicidal activity of the submerged macrophyte was evaluated.

Materials and Methods

Collection and Preparation of the Macrophytes Extract

Myriophyllum spicatum was collected from Terme Creek in Terme, Samsun, and brought to the laboratory in cold plastic containers. The macrophyte was weighed using a precision scale (100 g) and then ground with 250 mL of ethanol. The mixture was placed in a shaker and incubated at 100 rpm for 48 hours. After the incubation, the mixture was filtered through filter paper, and the ethanol was removed using an evaporator at 35°C. The resulting concentration of the extract was determined to be 48 µg/mL. Various concentrations of *M. spicatum* extract (1.5, 3, 6, 12, 24, and 48 µg/mL) were prepared by serial dilution with distilled water modified by Kaynak et al. (2019).

Culture of A. castellanii

A 1 mL suspension of *E. coli* (ATCC 25922) was prepared and added to the Ringer agar medium. Subsequently, 300 µL of *A. castellanii* (ATCC 30010) strain was added to the same medium and inoculated using a sterile pipette tip. After incubating the mixture at 26°C for 96 hours, the trophozoites were washed with Ringer Broth medium, collected, and assessed for both their count per mL and viability using a 0.4% trypan blue stain on a Thoma slide. The experiment began with an initial count of 2×10^6/mL trophozoites and 100% viability (Kaynak et al., 2019).

Determination of the Amoebicidal Activity Assays

Different concentrations of *M. spicatum* extracts (1.5, 3, 6, 12, 24, and 48 µg/mL) were placed in Eppendorf tubes, with 200 µL of each extract added to each tube. Following this, 200 µL of *A. castellanii* trophozoites was added to the corresponding Eppendorf tubes. A negative control (trophozoites + sterile distilled water) and a positive control (trophozoites + 0.05% Chlorhexidine gluconate) were also prepared. These tubes were incubated at 26°C for 24, 48, and 72 hours. At the end of each incubation period, 25 µL of 0.4% trypan blue was mixed with 25 µL of the parasite and *M. spicatum* extract solution and then incubated at room temperature for 5 minutes. Live and dead trophozoites were counted separately using a Thoma slide. The counts were performed three times (Kaynak et al., 2019).

Statistical Analysis

The data for changes observed at 24, 48, and 72 hours in test groups containing *M. spicatum* extracts were recorded using Microsoft Excel and IBM SPSS Statistics 27.0.1. The results are presented as a mean (Mean) ± standard deviation (SD). A 1% error margin was accepted in the comparison of "p" values (probability), and confidence intervals were set at 99% probability. The effectiveness of the extracts in terms of amoebicidal activity was assessed using post hoc multiple comparison analysis and Tukey pairwise comparisons in IBM SPSS Statistics 27.0.1. Logarithmic regression analysis was employed to determine the 50% inhibitory concentration (IC50) values for various *M. spicatum* extract concentrations that were lethal to trophozoites. These values were derived from logarithmic regression graphs. The percentage of cell death resulting from the amoebicidal effects of the *M. spicatum* extract concentrations at different time points was calculated in comparison to the control cells. Inhibitor concentrations producing 50% cell death were tested in three replicate experiments. Additionally, Principal Component Analysis (PCA) in Jamovi 2.4.11 was used to visualise explanatory variables and summarise the outcomes across all extract concentrations.

Results and Discussion

The viability of *Acanthamoeba* trophozoites was assessed during the experiment at 24, 48, and 72 hours at 26°C. Trophozoite growth was halted in *M. spicatum* leaf extract with an IC50/72h of 26.5 µg/mL (Table 1). More substantial inhibitory effects were observed at concentrations of 12, 24, and 48 µg/mL after 72 hours of exposure to the extract, showing significant activity against *Acanthamoeba* trophozoites. The *M. spicatum* extract exhibited lethal effects on most trophozoites at 48 and 24 µg/mL by the end of the 72 hours (Figure 1). The results were expressed as percentage inhibition with control cells (Figure 1). Among the different ethanolic *M. spicatum* extracts tested, the 1.5 µg/mL concentration exhibited the lowest anti-amoebic activity with a 72-hour IC50 value.

The results are presented as mean standard errors, as shown in Table 1.

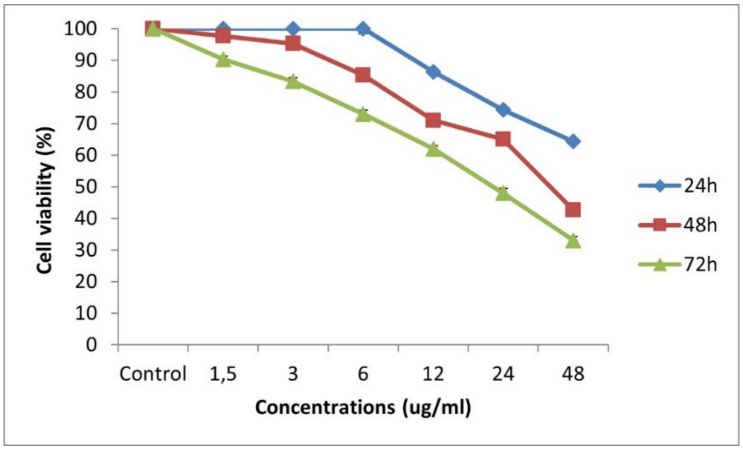

Figure 1. Amoebicidal effect of *M. spicatum* extract on *A. castellanii* trophozoites at different doses and time points

Tables 2 and 3 indicate which means are similar or different. Data are presented as mean ± SD. Statistically significant differences were observed between values marked with different letters, such as a and b. The statistical analysis revealed that the application time of the doses had a significant effect on viability, with $p<0.001$. Upon examining the impact of application times on viability, it was found that viability decreased as the exposure time increased. The highest viability was recorded after 24 hours, while the lowest viability was observed after 72 hours.

Furthermore, when the 48 µg/mL extract was compared at different time intervals, significant statistical differences in cell viability were observed between the 48-hour and 72-hour periods. Similarly, when the 24 µg/mL extract was compared across the three time points, significant differences in cell viability were observed between the 24-hour, 48-hour, and 72-hour measurements. For the 12 µg/mL concentration, comparisons across the three time intervals revealed statistically significant changes in cell viability at 24, 48, and 72 hours for all concentrations (Table 3).

To summarise the % viability data for all extract concentrations on A. castellanii trophozoites, Principal Component Analysis (PCA) was used to visualise the explanatory variables. The results revealed that the doses of 48, 24, and 12 µg/mL were negatively correlated with all other doses (1.5, 3, and 6 µg/mL), as well as the control group, with a clear separation on either side of the axis (Figure 2).

Aquatic macrophytes living underwater in natural rivers and stagnant waters are exposed to various abiotic stress factors, including solar radiation, temperature fluctuations, and mechanical stress caused by water flow. *M. spicatum* is an underwater plant that lives completely submerged, showing rapid growth and strong environmental adaptability. Due to its allelopathic effects, *M. spicatum* is a competitive species that exhibits rapid spreading strategies. It grows on all continents except Antarctica and is commonly found in colonies in eutrophic waters (Liu et al., 2019). Its strategy to thrive in eutrophic environments involves producing allelopathic chemical compounds. Several scientific studies have reported that *M. spicatum* produces chemical compounds that can inhibit the growth of cyanobacteria, showing cyanobacterialidal effects (Liu et al., 2007; Shao et al., 2009; Nakai et al., 2012; Jeong et al., 2021; Zuo et al., 2023).

The chemical composition of *M. spicatum* extract has been extensively studied, revealing a rich variety of bioactive compounds. In addition to polyphenols and fatty acids, the plant also contains alkaloids, terpenoids, and carotenoids, which have been linked to its allelopathic and antimicrobial properties. Specifically, *M. spicatum* has been reported to produce alkaloids such as *myriophylline*, which have shown potential as natural herbicides, aiding in the suppression of aquatic weed growth (Chen et al., 2017). Furthermore, carotenoids, including lutein and β-carotene, are present in the plant, contributing to its antioxidant activities and potential role in mitigating oxidative stress in aquatic environments (Li et al., 2020). The synergistic effects of these compounds may enhance the plant's ability to combat harmful algal blooms, particularly those caused by cyanobacteria, by interfering with their growth and photosynthetic activity (Liao et al., 2020). Studies have also highlighted the presence of saponins in *M. spicatum*, which are known for their antifungal and antimicrobial properties, further adding to its potential as a bioactive agent in aquatic ecosystem management (Zhao et al., 2018). The diversity of these compounds underscores the importance of *M. spicatum* as a natural source of biocides, offering promise for both environmental and pharmaceutical applications.

Table 1. The percentages of cell viability in *Acanthamoeba* trophozoites after being exposed to Various concentrations of *Myriophyllum spicatum* leaf extracts for 72 hours

The life cycle phase of *A. castellanii*	The different doses of *M. spicatum* leaf extracts	Cell viability percentage ± standard error (SE)
Trophozoites	48 µg/mL	33±1.20
	24 µg/mL	48±1.38
	12 µg/mL	62±0.92

Table 2. Mean ± SD values representing the percentages of cell viability of *Acanthamoeba castellanii* trophozoites following exposure to different concentrations of *Myriophyllum spicatum* ethanolic extract at different time intervals

Time (Hours)	Dose (µg/mL)	Mean±SD
24	Control	100.00±0.00[a]
	1.5	100.00±0.00[a]
	3	100.00±0.00[a]
	6	100.00±0.00[a]
	12	86.33±1.52[d]
	24	74.33±0.57[e]
	48	64.33±1.52[g]
48	Control	100.00±0.00[a]
	1.5	97.66±0.57[ab]
	3	95.33±1.15[b]
	6	85.33±1.52[d]
	12	71.00±1.73[f]
	24	65.33±1.15[g]
	48	42.66±2.51[i]
72	Control	100.00±0.00[a]
	1.5	90.33±1.52[c]
	3	83.33±2.08[d]
	6	73.00±2.64[ef]
	12	62.00±1.52[g]
	24	48.00±2.00[h]
	48	33.00±2.00[j]
p	<0.01	

The data are presented as mean ± SD. Statistical significance between values denoted by different letters, such as a and b, is considered meaningful

Table 3. Overview of the impact of *Myriophyllum spicatum* L. ethanol extract on the cell viability of *Acanthamoeba castellanii* trophozoites at different concentrations and exposure times (Mean ± SD)

Dose/Time	24 h	48 h	72 h	∑
Control	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}
1.5 µg/mL	100.00±0.00^{a}	97.66±0.57^{ab}	90.33±1.52^{c}	96.00±9.16^{b}
3 µg/mL	100.00±0.00^{a}	95.33±1.15^{b}	83.33±2.08^{d}	92.88±7.54^{c}
6 µg/mL	100.00±0.00^{a}	85.33±1.52^{d}	73.00±2.64^{ef}	86.11±11.80^{d}
12 µg/mL	86.33±1.52^{d}	71.00±1.73^{f}	62.00±1.52^{g}	74.55±9.16^{e}
24 µg/mL	74.33±0.57^{e}	65.33±1.15^{g}	48.00±2.00^{h}	63.88±9.80^{f}
48 µg/mL	64.33±1.52^{g}	42.66±2.51^{i}	33.00±2.00^{j}	48.33±12.29^{g}
∑	89.28±14.05^{a}	79.61±20.02^{b}	100.00±0.00^{a}	

Statistical differences were found between the values indicated as superscripts and those represented by different letters (a,b,…j = $p < 0.01$)

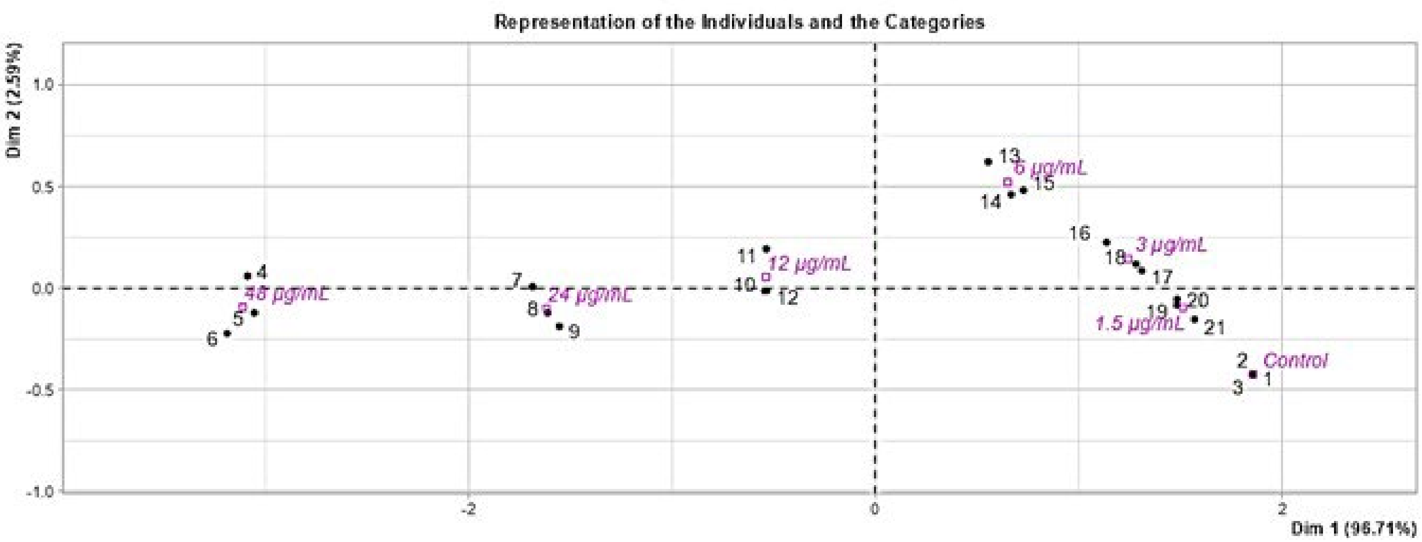

Figure 2. The PCA plot of *Myriophyllum spicatum* extract at various concentrations

M. spicatum produces significant amounts of fatty acids and polyphenols to suppress the cyanobacterium *Microcystis aeruginosa* (Nakai et al., 2012; Maredová et al., 2021). This helps reduce water turbidity by preventing excessive phytoplankton growth in eutrophic waters, facilitating the restoration, recolonisation, and regeneration of submerged macrophytes with proper light penetration (Švanys et al., 2014). Therefore, *M. spicatum* represents a promising natural source for preventing cyanobacterial harmful algal blooms (CyanoHABs) and can be used for the restoration of eutrophic water bodies by creating a cleaner aquatic environment. As a natural product, it could also be an important source for innovative drug development.

Allelochemicals released by aquatic plants containing secondary metabolites are distinguished by excellent biocompatibility, biological degradability, net algal inhibition, and minimal ecological harm (Zuo et al., 2023). However, different phytoplankton species show varying sensitivities to allelochemicals. For example, cyanobacteria and diatoms are more sensitive than green algae, while epiphytic species are less sensitive than phytoplankton. Additionally, environmental factors (such as light, temperature, and nutrients) can significantly affect the allelopathic impact of submerged macrophytes (Xi et al., 2009).

Acanthamoeba is an opportunistic protozoan commonly found in nature (mainly in water and soil). *Acanthamoeba* usually exists in two forms: trophozoites and cysts. Pathogenic species can lead to profound blindness due to *Acanthamoeba* keratitis (AK) and the rare granulomatous amoebic encephalitis (GAE), as well as skin and lung infections. In recent years, there has been an increase in *Acanthamoeba* infections, particularly AK. As a result, there has been growing scientific interest in the diagnosis, treatment, and prevention of *Acanthamoeba* infections (Wang et al., 2023).

To combat infections caused by *Acanthamoeba*, plant-based compounds may be utilised due to their bioactive molecular activities. By focusing on plants with antiparasitic properties, new drugs could be developed to treat *Acanthamoeba* infections (Mitsuwan et al., 2020).

With global warming, global water scarcity, and the reliance on household water tanks in developing countries, infections caused by free-living amoebas, such as Acanthamoeba, are expected to rise. Therefore, the development of new disinfectants that can effectively target pathogenic, free-living amoebas is a crucial issue (Siddiqui et al., 2022). Secondary metabolites obtained from natural sources are often considered to have more "drug-likeness and biological friendliness" compared to fully synthetic compounds, making them promising candidates for disinfectants, contact lens disinfectants, and drug development (Chin et al., 2006).

Aquatic plants (macrophytes) with anti-*Acanthamoeba* activity have rarely been studied. The ethanolic extract of the submerged aquatic plant *Ceratophyllum demersum* (coontail) was found to exhibit amoebicidal activity against *Acanthamoeba castellanii* trophozoites in an in vitro environment. After 72 hours of incubation, it was observed that the viability of trophozoites treated with *C. demersum* extract at concentrations of 30.4 and 60.8 µg/mL decreased to 42% and 58.33%, respectively (Taş et al., 2024).

No specific information is found in the current scientific literature regarding the amoebicidal (anti-amoebic) effect of *M. spicatum*. However, research on the antimicrobial and antioxidant properties of aquatic plants is increasing. It is known that aquatic plants, such as Ranunculus sphaerospermus, exhibit antibacterial and antioxidant properties (Ertürk et al., 2019). *M. spicatum* has antimicrobial effects (Ertürk et al., 2020), and *C. demersum* has anti-amoebic effects (Taş et al., 2024). Therefore, further research is needed to evaluate the potential anti-amoebic effects of *M. spicatum*. A study examining the in vitro antimicrobial effects of ethanol extracts from *M. spicatum* collected from two different locations on test organisms (four Gram-positive bacteria, four Gram-negative bacteria, and two fungi) found that the highest activity was observed against the Gram-positive bacterium *Bacillus subtilis* (inhibition zone diameter 24.50 ±6.24 mm/25 µL). The plant extract was more effective against Gram-positive bacteria compared to Gram-negative bacteria. Furthermore, the *M. spicatum* extract from the Miliç River (Samsun) was found to have a higher antimicrobial effect than the extract from Ulugöl Lake (Ordu) (Ertürk et al., 2020). The antimicrobial activity of *M. spicatum* suggests that it may also have potential anti-amoebic effects. In traditional medicine, *M. spicatum* is used externally as a wound-healing agent (Bolotova, 2015).

Although various phenolics, terpenes, and other phytochemicals have been isolated from *M. spicatum*, there is no literature on its pharmacological uses. At least 18 different phenolic compounds have been reported in *M. spicatum*, with ellagic acid, gallic acid, and various tannic acids being the most abundant compounds (Lambrechts et al., 2020).

Given the richness of these bioactive compounds, the plant presents potential for therapeutic applications, including antimicrobial and antiparasitic activities. One such area of interest may be the treatment of *Acanthamoeba* infections. That is why we examined the effect of *M. spicatum* on the trophozoite form of *Acanthamoeba. Acanthamoeba* infections are particularly challenging due to the parasite's ability to form cysts, which play a crucial role in its persistence and resistance to treatment. However, as noted, our study was specifically designed to evaluate the effects of *M. spicatum* on the trophozoite form of *Acanthamoeba*. Further studies are needed to assess the effects of the tested agents on various stages of the cyst life cycle.

Conclusion

The results indicated the amoebicidal activity of the ethanolic leaf extract of *M. spicatum* against *A. castellanii* trophozoites. It was observed that the parasite's survival rate decreased with increasing extract concentration. Additionally, when the impact of different application times on viability was analysed, it was found that the parasite's survival rate declined as the exposure time increased. Based on the findings, it can be inferred that the extract of *M. spicatum* could serve as a potential alternative or complementary treatment for Acanthamoeba infections under controlled conditions.

Compliance with Ethical Standards

Conflict of interest: The author declares no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: Ethics committee approval is not required for this study.

Data availability: The data will be made available upon request from the author.

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Disclosure: -

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