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In vitro, amoebicidal activities of submerged plant *Ceratophyllum demersum* L. extract against *Acanthamoeba castellanii* trophozoites

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ABSTRACT

Ceratophyllum demersum L. is a hydrophyte with potential for use as an analgesic, antipyretic and anti-inflammatory drug. It has also been stated that it is a hepatoprotective and anti-diarrheal agent with potential use in stomach disorders and respiratory diseases. Our study investigated the anti-amoebic activity of C. demersum, which became a hydrophyte underwater collected from Samsun River Miliç. Different concentrations of the pathogenic strain of *Acanthamoeba castellanii* (ATCC 30010) and the ethanolic extract of *C. demersum* were used to determine anti-Acanthamoeba activity. *A. castellanii* trophozoites were treated with *C. demersum* extract at different concentrations ranging from 1.9, 3.8, 7.6, 15.2, 30.4, 60.8 µg/mL and incubated at 26°C for 72 h. The 50% inhibitory concentration (IC50) of *C. demersum* extract was approximately 42.5 µg/mL at 72 hours. Trophozoite cell viability decreased to 42% and 58.33% in the presence of 30.4 and 60.8 µg/mL *C. demersum* extract at 72 hours, respectively. These results indicate that the ethanolic extract of *C. demersum* has anti-Acanthamoeba activity against *A. castellanii* trophozoites. The study highlights that this extract can be a potential protective drug source against *Acanthamoeba* trophozoites.

Keywords: *Ceratophyllum demersum, Acanthamoeba castellanii*, Amoebicidal activities, Hydrophyte

Introduction

Ceratophyllum demersum L. (Ceratophyllaceae), one of the important primary producers of aquatic ecosystems, is a cosmopolitan hydrophyte that lives completely under water (submerged), floats freely in the water column, does not produce roots, and can form dense covers just below the surface. The plant has a high ecological tolerance, often occurring in ponds, lakes, ditches, and calm streams with moderate to high nutrient levels. In Turkish waters, *C. demersum* and *C. submersum* are commonly found in the Yeşilırmak Delta (Taş & Topaldemir, 2021). The use of hydrophytes living in freshwater as medicinal plants has received less attention than terrestrial plants. However, phytochemical evaluations reveal the existence of secondary metabolites such as tannins, steroids, glycosides, flavonoids, phenolic compounds, and alkaloids. For this reason, researchers have recently shown interest in hydrophytes (Bhowmik et al., 2013; Roma et al., 2017).

C. demersum has traditionally been used to treat diarrhoea, fever, wounds, haemorrhoids or haemorrhoids, internal bleeding, hyperdipsia, and hematemesis (Li et al., 2020). Some studies have shown that *C. demersum* extracts are antioxidant (Karatas et al., 2015), antifungal (Malathy et al., 2015), insecticidal (Lu et al., 2007), antidiarrheal and wound healing (Taranhalli et al., 2011), antibacterial (especially Effective against *E. coli* and *Bacillus cereus*) and anti-leishmanial (Al-Halbosiy et al., 2020) activity. The literature research found no research on the anti-Acanthamoeba effects of *C. demersum.*

Acanthamoeba are opportunistic pathogens widely distributed throughout the world. *Acanthamoeba* spp. Commonly found in damp soil, freshwater accumulations, sewage, swimming pools, contact lens equipment, lakes, dam lakes, tap water, and air. They have two important diseases, Acanthamoeba keratitis (AK) and Acanthamoeba granulomatous encephalitis (GAE), caused by these parasites. GAE is usually chronic, lasting over a week, sometimes even months (Lass et al., 2014).

There are two forms in the life cycle of Acanthamoeba. It has an active trophozoite form with a dormant cyst under stressful conditions. Depending on the conditions, these two forms can transform into each other (Khan, 2006). The cyst wall of Acanthamoeba makes it tolerant to drugs. Changes in physiological and radiological conditions, chlorination and biocides do not prevent the survival of the cyst. Cysts are primarily responsible for the long-term treatment of Acanthamoeba infections. Cysts also resist the drugs used (Elsheikha et al., 2020).

Acanthamoeba cysts are difficult to treat because they are highly resistant to antibiotics and other agents. Therefore, studies are ongoing to find an effective treatment against Acanthamoeba infections (Chiboub et al.,2017). This study investigated the antiparasitic effect of *C. demersum,* a submerged hydrophyte collected from Samsun River Miliç. The present study aimed to survey and evaluated in vitro antiamoebic activities of C. demersum, a submerged hydrophyte collected from Samsun River Miliç.

Materials and Methods

Sample Collection

Aquatic plant species (*C. demersum*) were collected from Miliç River, Terme, Samsun (Figure 1). Collected samples were washed with water to remove epiphytes and other freshwater organisms. The plants were transported to the laboratory in sterile polythene bags.

Extraction of Macrophytes

The collected *C. demersum* samples were moved in a cool container to the laboratory. Firstly, the samples were washed with distilled water. Then, the samples were shade-dried, cut into small pieces, and finely powdered in a mixer grinder. An organic solvent (ethanol) was used for extraction. Ethanol has proven to be the best solvent for extracting compounds with antimicrobial activity and antioxidant capacity (regardless of the extraction method used) (Borges et al., 2020). They were homogenized with a blender. The samples (30 g) were extracted in 250 mL of ethanol for 48 h at room temperature in a shaking incubator. The solvent was then removed from the aquatic plant extract by evaporation. The final concentration was adjusted to 60.8 µg/mL with distilled water from the residue. Different dilutions of *C. demersum* extract (1.9, 3.8, 7.6, 15.2, 30.4, and 60.8 µg/mL) were made by serial dilution with distilled water.

Amoebicidal Activity

Acanthamoeba castellanii (ATCC 30010 from the American Type Culture Collection) was used in this study. The A. castellani strain was cultured on Ringer agar plates seeded with Gram-negative bacteria (*E. coli*) as a food source. The plates were incubated at 26 ºC in the incubator, and three days later, they were microscopically examined for the presence of *Acanthamoeba* trophozoites (Tepe et al., 2012; Koloren et al., 2019).

The pathogenic strain of *A. castellanii* (ATCC 30010) and the different concentrations of *C. demersum* ethanolic extract were used to determine the anti-amoebic activity assays. *A. castellanii* trophozoites were treated with different concentrations of *C. demersum* extract in the range of 1.9, 3.8, 7.6, 15.2, 30.4, and 60.8 µg/mL and incubated for different hours at 26 ºC. The increase or decrease in amoebae was checked at 1, 3, 6, 8, 24, 48, and 72 h intervals using a Thoma haemocytometric chamber. Approximately 100 *A. castellanii* trophozoites were examined each time, and all the tests were repeated three times. The control group was a culture of amoebae without *C. demersum* extract and statistical analyses were done to show the cell viability percentage.

Figure 1. *Ceratophyllum demersum* collected from Miliç River (Terme, Samsun)

Statistical Analysis

A one-way test of variance (ANOVA) with the SPSS software package for Windows was applied to complete all statistical analogies was used for all results. The results were expressed as Mean ± standard error (SE). We found differences in the means by performing a Tukey multiple comparison analysis to determine which means are similar or different. Analysis of variance was determined by A one-way test of variance (ANOVA) with the SPSS software package for Windows. Differences at $p < 0.01$ were statistically significant.

Moreover, Principal Components Analysis (PCA) was carried out with the Jamovi 2.4.11 program.

Results and Discussion

This study examined different ethanolic extracts of C. demersum for their anti-amoebic activity against *A. castellanii* trophozoites at different hours at 26 ℃.

The amoebicidial activity of *C. demersum*'s ethanolic extracts on A. castellanii trophozoites is shown in Table 1 and Figure 1.

The trophozoite growing stopped in ethanolic extracts of *C. demersum* with IC50/72h at 42.5 µg/mL. The ethanolic extracts of *C. demersum* showed strong inhibitory effects at 60.8, 30.4, and 15.2 µg/mL concentrations at 72 h against Acanthamoeba trophozoites. The seaweed extract (1.9 µg/mL) on trophozoites with IC50/72 h out of the different concentrations of ethanolic extracts of C. demersum used in the study showed the most vital anti-amoebic activity.

The results were expressed as percentage inhibition relative to control cells (Figure 2).

The results are the mean standard errors, as in Table 1. Tables 2 and 3 show which means are similar and different.

The data were expressed as mean ± SD. The statistical difference between values marked with different letters, such as a and b, is important.

It was determined that the effect of the application times of the doses on viability was statistically significant, $p<0.001$. When the effects of application times on viability were examined, it was determined that viability decreased as the waiting time increased. The highest viability was measured at the end of 24 hours, while the lowest viability was measured at the end of the 72nd hour.

Explanatory variables were visualised with PCA to summarize the results for all doses. Figure 3 shows the PCA biplot of the values of different doses at different times. Both the first (F1, 2.31%) and second (F2, 97.31%) principal components collectively contributed to the largest variation (99.6%) in the dataset.

The % cell viability values in the 24 hours of all applied doses show a significant negative correlation with the 72-hour values, p (0.05). The 48th-hour cell viability data are located in the middle of the axis, and it has been observed that cell viability decreases after this time. In this context, the 48th hour can be considered the threshold value for all doses (Figure 4).

Table 4 shows that all axes explain 99.6% of the variances.

Table 1. Percentages of cell viability in the Acanthamoeba trophozoites when exposed to the different concentrations of Ceratophyllum demersum, extracts at 72h.

The stage of A. castellanii	The concentrations of C. demersum leaf extracts	Percentage of cell viability ±SE
Trophozoites	60.8 µg/mL	41.67 ±1.20
	30.4 µg/mL	58 ±1.53
	15.2 µg/mL	63 ±0.58

Table 2. Mean ± SD values for the percentages of cell viability of Acanthamoeba trophozoites when exposed to various concentrations of *Ceratophyllum demersum* L. ethanolic extract at varied hours

Hour	Dose	Percentages of cell viability
24	Control	99,33±1,15^{a}
	1.9 µg/mL	99±1,73^{ab}
	3.8 µg/mL	96,33±1,53^{abc}
	7.4 µg/mL	94,33±0,58^{bc}
	15.2 µg/mL	93±1^{cd}
	30.4 µg/mL	89±1de
	60.8 µg/mL	79±1^{f}
48	Control	99±1^{ab}
	1.9 µg/mL	97,67±2,52^{abc}
	3.8 µg/mL	89±1de
	7.4 µg/mL	85±1^{e}
	15.2 µg/mL	80±1^{f}
	30.4 µg/mL	73±1^{g}
	60.8 µg/mL	59±1^{hi}
72	Control	97,33±0,58^{abc}
	1.9 µg/mL	87±1^{e}
	3.8 µg/mL	79,33±3,06^{f}
	7.4 µg/mL	71±1,73^{g}
	15.2 µg/mL	63±1^{h}
	30.4 µg/mL	58±2,65^{i}
	60.8 µg/mL	41,67±2,08^{j}
p	<0,001	

Table 3. Summary of the effect of Ceratophyllum demersum L. ethanol extract on cell viability of Acanthamoeba trophozoites at various doses and durations (Mean±SD)

	24	48	72	Σ
Control	99,33±1,15^{a}	99±1^{ab}	97,33±0,58^{abc}	98,56±1,24^{a}
1.9 µg/mL	99±1,73^{ab}	97,67±2,52^{abc}	87±1^{e}	94,56±5,92^{b}
3.8 µg/mL	96,33±1,53^{abc}	89±1de	79,33±3,06^{f}	88,22±7,6^{c}
7.4 µg/mL	94,33±0,58^{bc}	85±1^{e}	71±1,73^{g}	83,44±10,22^{d}
15.2 µg/mL	93±1^{cd}	80±1^{f}	63±1^{h}	78,67±13,06^{e}
30.4 µg/mL	89±1de	73±1^{g}	58±2,65^{i}	73,33±13,51^{f}
60.8 µg/mL	79±1^{f}	59±1^{hi}	41,67±2,08^{j}	59,89±16,23^{g}
Σ	92,86±6,79^{a}	83,24±13,44^{b}	71,05±17,87^{c}	

Data were expressed as mean ± SD

A statistical difference exists between values marked with letters such as a and b.

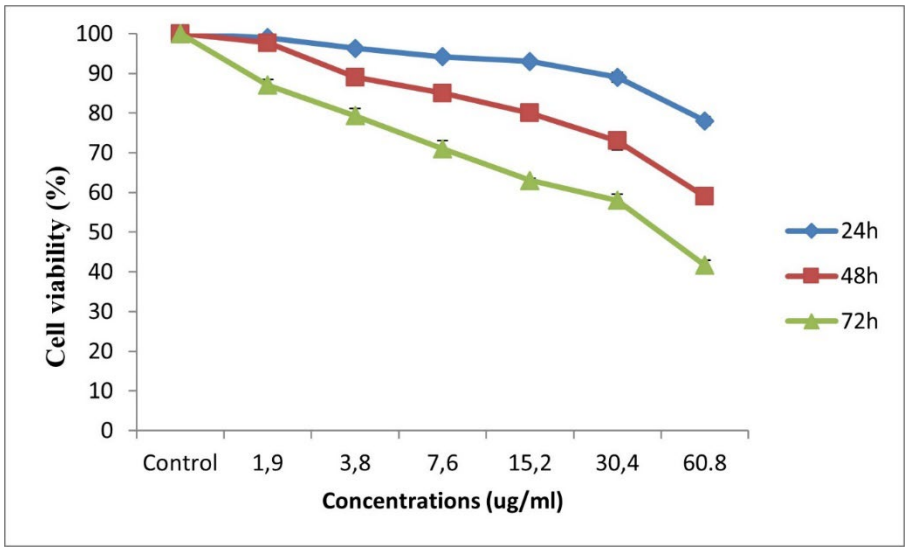

Figure 2. The effect of various concentrations of *Ceratophyllum demersum* L. ethanolic extract on the proliferation of *A. castellani* trophozoites at different hours

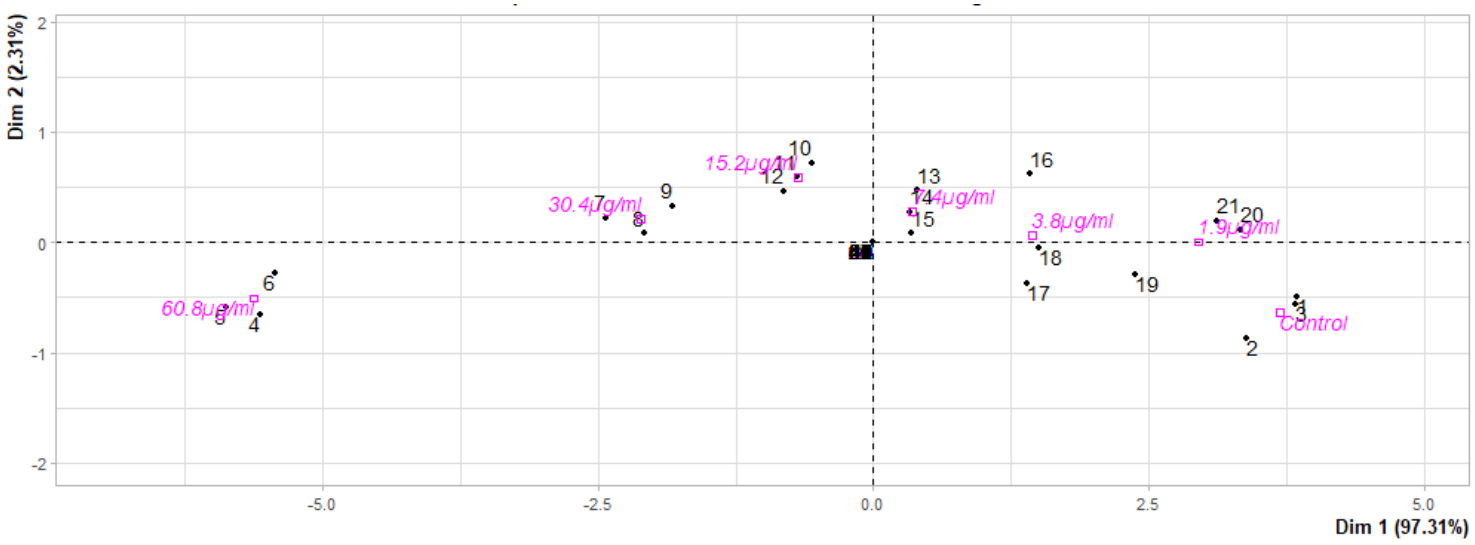

Figure 3. Representation of the individuals (and the categories)

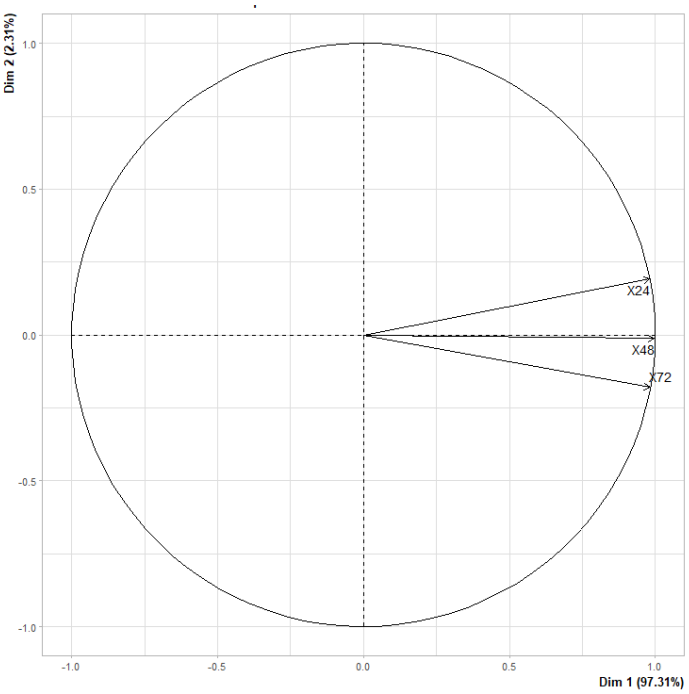

Figure 4. Representation of the active variables

Table 4. Eigenvalue and (Cumulative) percentage of variance

	Eigenvalue	% of the variance	Cumulative %
Dim. 1	2.9193	97.311	97.3
Dim. 2	0.0692	2.308	99.6

Awati et al. (2020) reported that the original phytoconstituents components of *C. demersum* are flavonoids, alkaloids, sterols, proteins, tannins, quercitrin, and volatile oil. Many studies have been reported on *C. demersum* L. because it is antidiarrhoeal and wound curative activity (Ashok et al., 2011; Brunton et al., 2006), antioxidant and anti-acetyl cholinesterase potential (Monferran et al., 2007; Lone et al., 2023), analgesic activity (Karale et al., 2013), anti-inflammatory and antineoplastic potential (Kurashov et al., 2016) anti-ulcer activity (Parmar et al.,1993; Niesink et al., 1996), anti-pyretic activity (Karale et al., 2013; Kurashov et al., 2016).

Extract from *C. demersum* has a high concentration of phytol, according to a study by Rashid et al. in 2023. Phytol has several beneficial properties, such as anti-inflammatory, antimicrobial, anticancer, and diuretic effects (Beulah et al., 2018; Ramya et al., 2021). Other studies have also discovered that phytol has anxiolytic, anticonvulsant, antinociceptive, cytotoxic, antioxidant, immune-modulating, and metabolism-modulating properties (Islam et al., 2018). Plant extracts have also been found to contain Vitamin E, as reported by Rashid et al. in 2024. Vitamin E has several health benefits, including antioxidant, antiaging, analgesic, antidiabetic, anti-inflammatory, antidermatitic, anticancer, antispasmodic, and hepatoprotective activities. Studies conducted by Nisha in 2018, Mujeeb et al. in 2014, and Ramya et al. in 2021 have confirmed these benefits.

According to our search, several in vitro studies have been performed on the effectiveness of *C. demersum L.* in treating anti-microbial activities. The antimicrobial effect of *C. demersum* extract using acetone, butanol, and methanol on *Staphylococcus aureus* and *Escherichia coli* and *Aspergillus niger* was investigated (Fareed et al., 2008; Malathy & Shaleesha, 2015). The antibacterial potential of different solvent extracts obtained from a free-floating aquatic plant, C. demersum, on fish bacterial pathogens was performed by Lone et al. (2023). One report was found on the effect of the methanol extract of *C. demersum* as an anti-leishmanial. The methanol extract of *C. demersum* in quantities of 25, 50, 100, 200, 400, and 800 µg/mL was investigated for its effect on an anti-leishmanial, anticancer, and antibacterial. The concentration of 800 µg/mL of *C. demersum* has been shown to have significant activity against Leishmania.

Although many studies in Türkiye show the amoebacidal effects of plant extracts on *A. castellanii* (Malatyali et al.,2012a; Malatyali et al.,2012b; Degerli et al., 2012a; Degerli et al., 2012b; Tepe et al., 2012; Kaynak et al., 2018; Kaynak et al., 2019; Koloren et al., 2019) have been reported, according to the internet search no report was found on the anti-*Acanthamoeba* effect of ethanol extract of *C. demersum*.

Despite the availability of a small number of chemotherapeutic agents for anti- *Acanthamoeba* therapy, the management of patients with AK, and GAE in particular, remains in great difficulty. Only in patients presenting early combined treatments may be promising. Furthermore, long-term use of the main drugs used in the treatment of AK brings other problems. Additionally, treatments can cause toxic keratopathy, encystation, and the development of resistant *Acanthamoeba* cysts. Recent studies suggest that microbial co-infections should be considered in cases of AK where *Acanthamoeba* therapy is ineffective. Co-infections make treatment difficult,

and other supportive treatment methods are needed. This situation further complicates treatment regimens and requires additional therapeutic interventions (Elsheikha et al., 2020).

The study found that animals treated with aqueous and methanolic extract of *C. demersum* had faster wound contraction and rate of epithelialization in the excision wound model. This may be attributed to phytoconstituents like tannins and flavonoids, which promote wound healing due to their astringent and antimicrobial properties (Manjunath et al., 2005; 2007). Based on the above discussion, it can be concluded that both extracts of *C. demersum* have potent antidiarrheal and wound-healing activities. Furthermore, the study found that the extracts were equally effective as the standard drugs used for these purposes (Taranhalli et al., 2011). Various bioactive compounds in these plant extracts align with their traditional use for medicinal purposes in various cultures (Rashid et al., 2023).

This study aims to shed light on the effectiveness of this extract against *Acanthamoeba* trophozoites and to pave the way for potential applications as candidates for preventing infection with the parasite. The findings of this study are promising and highlight the potential of *C. demersum* as a natural medicine against acanthamoeba infections. Results indicated that ethanolic extract of *C. demersum* showed anti-*Acanthamoeba* activity against *A. castellanii* trophozoites, especially in higher concentrations. These results indicate that the aquatic macrophytes used for medical, pharmacological, biological, or environmental purposes also have an antiparasitic effect.

Conclusion

Aquatic plants have antimicrobial and antiparasitic effects and have been used for medical, pharmacological, biological, and environmental purposes. Although not pure antibiotic substances, plant extracts have significant effects (Ertürk et al., 2019). Recent studies have shown that the aquatic plant *C. demersum* contains bioactive compounds with great pharmacological potential and could contribute to the development of future drugs (Rashid et al., 2023). Hydrophytes are a natural resource that can be considered medicinal plants, and further research is required to isolate and characterize the phytochemical components of extracts obtained from these plants using different extraction methods. Developing plant-based therapeutics is a promising area that requires further research and development.

Compliance with Ethical Standards

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

Ethics committee approval: This study does not require ethics committee permission or any special permission.

Data availability: Data will be made available on request.

Funding disclosure: -

Acknowledgements: -

Disclosure: -

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