



***In vitro* amoebicidal and antioxidant activity of *Helianthemum ledifolium* (L.) Mill. varieties**

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Abstract

Purpose: *Acanthamoeba* spp. are opportunistic protozoa that cause *Acanthamoeba keratitis* (AK), an ocular infection frequently associated with the use of contact lenses. Given the limitations and adverse effects of current treatments, this study aimed to discover natural, safe, and cost-effective therapeutic alternatives. Specifically, the purpose was to investigate the *in vitro* amoebicidal and antioxidant activities of methanol and water extracts from three varieties of *Helianthemum ledifolium* (L.) Mill.

Method: The study evaluated methanol and water extracts obtained from the aerial parts of three varieties of *H. ledifolium* (*H. ledifolium* var. *ledifolium*, *H. ledifolium* var. *microcarpum*, and *H. ledifolium* var. *lasiocarpum*). Amoebicidal activity was tested *in vitro* against *Acanthamoeba castellanii*. In addition, total phenolic and flavonoid contents were quantified using Folin-Ciocalteu and AlCl₃ reagents, respectively. Antioxidant activities were evaluated by measuring radical scavenging activities using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) assays.

Findings: The amoebicidal activity against *Acanthamoeba castellanii* showed a dose- and time-dependent effect. Methanol extracts exhibited greater amoebicidal potency compared to water extracts, even at lower concentrations, and also have the highest total phenolic and flavonoid contents in relation to antioxidant radical scavenging activities. The most potent toxic effect was observed in *H. ledifolium* var. *lasiocarpum* methanol extract, with an LD₅₀ of 0.0034 mg/mL at 72 hours. *H. ledifolium* var. *microcarpum* methanol extract (MicM) exhibited the fastest amoebicidal effect (LT₅₀ = 0.0476 h at 8 mg/mL).

Conclusion: These results suggest that *H. ledifolium* represents a promising candidate for the development of natural antiparasitic agents. The superior amoebicidal activity of methanol extracts is strongly related to their significantly richer total phenolic and flavonoid contents compared to water extracts, indicating that methanol is a more effective solvent for obtaining potential bioactive compounds. *H. ledifolium* taxa have the potential to be an important natural resource for future antiparasitic drug research, especially in the context of increasing drug resistance and treatment failure.

Keywords: Amoebicidal activity, Antioxidant activity, *Helianthemum ledifolium*, *Acanthamoeba castellanii*

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Helianthemum ledifolium* (L.) Mill. varyetelerinin *in vitro* amibisidal ve antioksidan Aktivitesi*Özet**

Amaç: *Acanthamoeba* türleri, sıklıkla kontakt lens kullanımıyla ilişkili bir göz enfeksiyonu olan *Acanthamoeba keratitine* (AK) neden olan fırsatçı protozoalardır. Mevcut tedavilerin sınırlamaları ve yan etkileri göz önüne alındığında, bu çalışma doğal, güvenli ve uygun maliyetli terapötik alternatifleri keşfetmeyi amaçlamıştır. Özellikle, amaç *Helianthemum ledifolium* (L.) Mill'in üç varyetesinden elde edilen metanol ve su ekstralarının *in vitro* amibisidal ve antioksidan aktivitelerini araştırmaktır.

Yöntem: Çalışmada, *H. ledifolium* varyetelerinin (*H. ledifolium* var. *ledifolium*, *H. ledifolium* var. *microcarpum* ve *H. ledifolium* var. *lasiocarpum*) toprak üstü kısımlarından elde edilen metanol ve su ekstraları değerlendirilmiştir. Amibisidal aktivite *Acanthamoeba castellanii*'ye karşı *in vitro* test edilmiştir. Toplam Fenolik ve flavonoid içerikleri sarsasıyla Folin-Ciocalteu reaktifi ve AlCl₃ reaktifi kullanılarak belirlenmiştir. Antioksidan aktiviteleri ise DPPH (2,2-Difenil-1-pikrilhidrazil) ve ABTS (2'-Azino-bis (3-etilbenzotiyazolin-6-sülfonik asit) diamonyum tuzu) analizleri ile radikal temizleme aktiviteleri ölçülerek değerlendirildi.

Bulgular: *Acanthamoeba castellanii*'ye karşı amip öldürücü aktivite doz ve zamana bağlı bir etki gösterdiği görüldü. Metanol ekstraları, daha düşük konsantrasyonlarda bile, su ekstralarına kıyasla daha yüksek amip etki gösterdi. Ayrıca antioksidan radikal temizleme aktiviteleri ile ilişkili olarak yüksek toplam fenol ve flavonoid içeriğine sahiptir. En güçlü toksik etki, 72 saatte 0,0034 mg/mL'lik bir LD₅₀ ile *H. ledifolium* var. *lasiocarpum* metanol ekstresinde (LasM) gözlemlendi. *H. ledifolium* var. *microcarpum* metanol ekstresi (MicM) en hızlı amibisidal etkiyi göstermiştir (LT₅₀ = 0,0476 h, 8 mg/mL'de).

Sonuç: Bu sonuçlar *H. ledifolium*'un doğal antiparaziter ajanların geliştirilmesi için umut verici bir aday olduğunu göstermektedir. Metanol ekstralarının yüksek amibisidal aktivitesi, su ekstralarına kıyasla önemli ölçüde daha zengin toplam fenolik ve flavonoid içerikleriyle güçlü bir şekilde ilişkilidir ve bu sonuç metanolün potansiyel biyoaktif bileşikleri elde etmek için daha etkili bir çözücü olduğunu göstermektedir. *H. ledifolium* taksonları, özellikle artan ilaç direnci ve tedavi başarısızlıkları bağlamında, gelecekteki antiparaziter ilaç araştırmaları için önemli bir doğal kaynak olabilir potansiyeline sahiptir.

Anahtar kelimeler: Amibisidal aktivite, Antioksidan aktivite, *Helianthemum ledifolium*, *Acanthamoeba castellanii*

1. Introduction

Protozoa are unicellular microorganisms typically too small to be observed without a microscope. They are ubiquitous in terrestrial and aquatic environments, including both freshwater and saline habitats. They have a structure that can perform all life events independently. *Acanthamoeba* spp. are free-living opportunistic protozoa commonly found in the environment, occurring in both vegetative trophozoite and resistant cyst stages throughout their life cycle. The trophozoite form lives on various bacteria and turns into the cyst form when environmental conditions become unfavorable [1,2]. Additionally, this organism has been isolated from diverse environments, including swimming pools, tap water, mineral water bottles, and even contact lens cleaning solutions. *Acanthamoeba keratitis* (AK) and *Acanthamoeba granulomatous encephalitis* are two well-known conditions caused by this organism. AK is a sight-threatening corneal infection caused by free-living amoebae of the genus *Acanthamoeba* spp. [3]. The most frequently reported clinical symptoms of AK include severe pain, eye redness, sensitivity to light, swelling, perforation, loss of epithelial tissue, and vision impairment. The primary clinical feature of AK is the presence of a stromal infiltrate in the shape of a ring. AK is also closely linked to contact lens use, and inadequate hygiene and other wrong lens-related practices are highlighted as important contributing factors. Widespread use of therapeutic agents for the treatment of AK can sometimes cause adverse reactions in patients. For this reason, researchers have explored alternative treatment methods for AK [4]. Medicinal plants are utilized to develop new agents with high efficacy and low toxicity for enhancing therapy. Essential oils and plant extracts have demonstrated effectiveness in inhibiting the proliferation of diverse microorganisms, including bacteria, parasites, yeasts, and filamentous fungi [5]. Plant extracts have been identified as potent inhibitors of various microbial agents such as bacteria, parasites, and fungi. Medicinal plants and herbal extracts have been studied as potential sources of amoebicidal agents for *Acanthamoeba* [4]. Researchers continue to investigate natural products, particularly phenolic rich plant extracts, as a source of novel amoebicidal candidates. In Hamad (2023) *in vitro* screening study, several newly proposed plant extracts were evaluated side-by-side with commonly used biocides against *Acanthamoeba castellanii* trophozoites and cysts. The study highlighted that an accurate appraisal of amoebicidal potential requires assays encompassing both life stages, together with host-cell safety considerations [6].

One study demonstrated that *Iris setosa* Pall. ex Link extract exhibits marked amoebicidal activity against *Acanthamoeba castellanii* and *A. polyphaga* [7]. In another study, *Ceratophyllum demersum* L. was evaluated *in vitro* and showed amoebicidal activity against *A. castellanii* trophozoites [8]. Another study looked at ethanolic extracts of *Sambucus ebulus* L. and assessed how effective they were at killing amoebicidal activity [9]. Root extracts of

Onopordum acanthium L. were tested *in vitro*, and amoebicidal outcomes were reported together with cytotoxicity measurements [10].

In previous scientific studies on *Helianthemum* Miller (Cistaceae) species, it has been reported that the high biological activities of plants are due to the presence of polyphenolic compounds. For example, scientific studies have demonstrated that they exhibit important pharmacological effects, including, cytotoxic [11], antioxidant [12], antimicrobial [11], antiprotozoal [13], and amoebicidal [4] activities.

The largest genus in the Cistaceae family, *Helianthemum* Miller, contains approximately 110 heliophytic species of shrubs, subshrubs, and annual herbs. It is widely distributed throughout Europe, America, North Africa, and Central Asia on marl, gypsum, limestone, sand dunes, coastal scrub, steppe, macchie, and rocky slopes [14]. *Helianthemum ledifolium* (L.) Mill. is an erect annual reaching up to 60 cm in height. Leaves are oblong, 1–5 × 0.4–1.2 cm, and the bracts are usually stipulate. Flowers are typically subsolitary in leafy racemes or arranged in long, lax, sometimes branched inflorescences with 5–10 flowers per axis. Pedicels remain erect in both flower and fruit and are equal to or shorter than the calyx. Sepals are acuminate, and the fruit is a capsule, 4–12 mm long [15].

In this study, *in vitro* amoebicidal and antioxidant activities of water and methanol extracts prepared from aerial parts of *Helianthemum ledifolium* varieties were investigated for the first time. These varieties include: *H. ledifolium* (L.) Miller var. *ledifolium* (L.) Miller (Led); *H. ledifolium* (L.) Miller var. *microcarpum* Willk. (Mic); and *H. ledifolium* (L.) Miller var. *lasiocarpum* (Willk.) Bornm. (Las). In this work, we evaluated the efficacy of methanol and water extracts of *H. ledifolium* taxa against *Acanthamoeba castellanii* trophozoites and cysts. Furthermore, the phenolic compositions of these extracts were quantified, and their antioxidative properties (DPPH•+ and ABTS•+ radical scavenging activities) were evaluated comparatively.

2. Materials and methods

2.1. Plant material

Helianthemum ledifolium varieties were collected from Türkiye. The plant materials were deposited at Hacettepe University, Faculty of Science Herbarium, Ankara, Turkey.

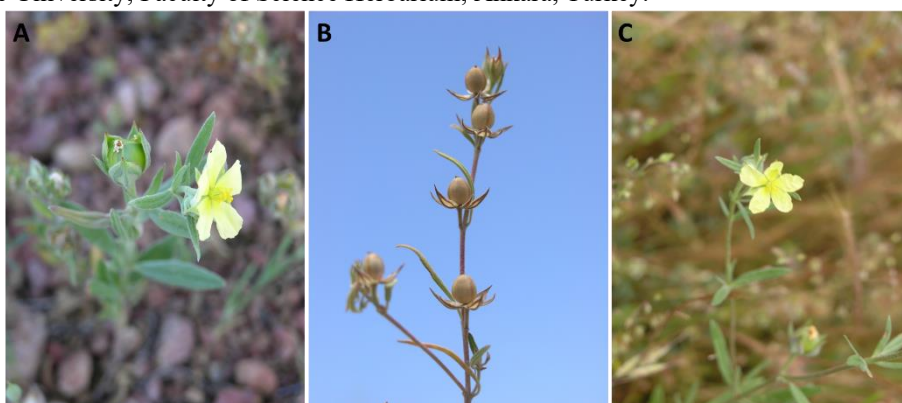


Figure 1. Photographs and herbarium codes for (A) *H. ledifolium* var. *ledifolium* (EBY-1191), (B) *H. ledifolium* var. *microcarpum* (EBY-1557), (C) *H. ledifolium* var. *lasiocarpum* (EBY-975). (Photographed by Dr. Emine Burcu Yeşilyurt)

The *Helianthemum ledifolium* specimens used in this study were collected from the following localities: *H. ledifolium* var. *ledifolium* (B4: Ankara, Beytepe Campus, slope, 980 m), *H. ledifolium* var. *microcarpum* (B4: Kahramanmaraş, Göksun, Ahmetli Village, oak clearings, 1344 m) and *H. ledifolium* var. *lasiocarpum* (B1: Between Çatıldere and Foça, Çoraklar road, maquis, 15 m).

2.2. Plant Extraction

In the study, aerial parts of *H. ledifolium* varieties were ground into small pieces using a mechanical grinder. After, water and methanol extracts were prepared by applying the maceration method to the plant samples at room temperature. The extract were filtered, and the solvents were added again. This process continued for three days. The filtrates were combined and concentrated using a rotary evaporator. All extracts are stored at -20°C until the experiment. Water and methanol extracts were prepared from these aerial parts and coded systematically as follows: Led water extract (LedW), Led methanol extract (LedM), Mic water extract (MicW), Mic methanol extract (MicM), Las water extract (LasW), and Las methanol extract (LasM).

2.3. Total Phenol Contents of The Extracts

The total phenol contents (TPC) of all extracts were determined using the Folin-Ciocalteu method as equivalent to gallic acid [16]. For the assay, 6 mL of distilled water, 100 µL of sample solution, and 500 µL of Folin-Ciocalteu reagent were prepared. After 1 minute, 1.5 mL of 20% aqueous Na₂CO₃ was added, followed by the addition of 10 mL of distilled water. A reagent blank without the sample was used as a control. Incubation was performed at 25°C for 2 hours. Then, the absorbance values were measured at 760 nm and compared with the gallic acid calibration curve. The total phenol amounts of the extracts were calculated as equivalent to gallic acid (GAE). The results were given as mean values ± standard deviation.

2.4. Total Flavonoid Contents (TFC) of The Extracts

The total flavonoid contents (TFC) of all extracts were assessed according to Sun et al. (2011) [17]. Briefly, 20 µL of each sample (1 mg/mL) or reference compound (quercetin) was mixed with 30 µL of 5% sodium nitrite. After 6 min, 50 µL of a 10% AlCl₃ solution was added, and the mixture was kept untouched for 5 min. Next, 100 µL of a 10% NaOH solution was added, the mixture was incubated at 25°C for 15-20 min, and the absorbance values were measured at 510 nm. TFC was expressed as quercetin equivalents (QUE). The results were given as mean values ± standard deviation.

2.5. DPPH• Radical Scavenging Activity

Antioxidant activity of all extracts were determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method [18]. In this method, a certain proportion of water and methanol extracts is added to the DPPH solution and incubated in the dark for 2 hours. Each sample was tested a concentration range of 1000 to 31.25 µg/mL. Then, the color change in the DPPH solution and the absorbance measurements at 517 nm are evaluated. The experiments were evaluated three times, and the mean values were given. In this study, ascorbic acid was used as reference standards for comparison.

2.6. ABTS• Radical Scavenging Activity

The free radical scavenging capacity of plant extracts was also studied using the ABTS radical cation decolorization assay [19], which is based on the reduction of ABTS radicals by antioxidants of the plant extracts tested. ABTS^{•+} was dissolved in deionized water to a 7mM concentration. ABTS radical cation (ABTS^{•+}) was generated by reacting ABTS solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. For the assay, the ABTS^{•+} solution was diluted in deionized water or ethanol to an absorbance of 0.7 ± 0.02 at 734 nm. An appropriate solvent blank reading was recorded. Then, with the addition of 100 µL of water or ethanolic (according to solubility) plant extract solutions to 3 mL of ABTS^{•+} solution, the absorbance values were taken at 30°C, 10 min after initial mixing. All solutions were used on the day of preparation, and all determinations were carried out in triplicate. In this study, ascorbic acid was used as reference standards for comparison. Each sample was tested a concentration range of 1000 to 31.25 µg/mL.

2.7. Antiparasitic Activity Studies

In the study, *A. castellanii* from Cumhuriyet University Parasitology Laboratory and *Escherichia coli* strains from Ordu University Faculty of Arts and Sciences Biology Department were taken and cultured to ensure continuity [20].

2.8. Culture of *in vitro* *Acanthamoeba castellanii*

The prepared media were diluted with 0.5 ml of Page's solution, and the 24-hour *E. coli* isolate was spread on agar. Samples taken from *A. castellanii* isolate were inoculated into the medium. The parasites were cultured for 72 hours at 26°C before the intact trophozoites were removed from the petri dish, washed with Page's solution, and centrifuged at 1500 x g for 5 minutes. 4% trypan blue was used to count the trophozoites on the hemocytometry slide to assess their vitality. In an Eosin Methylene-blue agar (EMB) medium that had been made under the usage procedure, *E. coli* was grown. In the test, Page's amoeba saline solution was used. The prepared solution was kept at 4°C after being autoclave sterilized at 121°C for 15 minutes. After being heated in 100 ml of Page's solution to dissolve 1.5 g of agar, the mixture was autoclaved at 121°C for 15 minutes before being poured into petri dishes. Before testing, prepared media were kept at 4°C. The prepared media were diluted with 0.5 mL of Page's solution, and 24-hour *E. coli* isolate was spread onto the agar. Samples taken from *A. castellanii* isolate were inoculated into the medium. The parasites were cultured for 72 hours at 26 °C before the intact trophozoites were removed from the petri dish, washed with Page's

solution, and centrifuged at 1500 x g for 5 minutes. 4% trypan blue was used to count the trophozoites on the hemocytometry slide to assess their vitality [2].

2.9. Amoebicidal Activity Assays

The extracts were prepared in 0.9 % saline at concentrations ranging from 1 to 32 mg/mL, and 200 µL of each was then dispensed into sterile Eppendorf tubes. After adjusting the *A. castellanii* concentration to 30×10^6 trophozoites/ml, 200 µL was added to the tubes, and they underwent an incubation period at room temperature. *A. castellanii* (20×10^6 parasites/ml) with 98% viability was added to 200 µL each and incubated at room temperature. The viability of the parasite was checked and recorded at certain times. Fluconazole was used as the standard control drug.

2.10. Statistical Analysis

Mean and standard deviation (SD) were used to describe the data set. Amoebicidal assays were conducted on *A. castellanii*. The survival rates obtained were analyzed using Two-Way Analysis of Variance (ANOVA) to determine the interaction and main effects of dose and exposure time. Following the analysis, significant differences between groups are given in the supporting information. The relationship between doses and exposure times is shown in SI Table 1, and the relationship between exposure times is shown in SI Table 2. The Lethal Doses (LD₅₀, LD₉₀), and Lethal Time (LT₅₀, LT₉₀) values for given periods were calculated using probit analysis. The cutoff for statistical significance was set at $p < 0.05$. SPSS 22, a statistical program, was utilized for all analyses. DPPH and ABTS radicals scavenging activities were statistically significant, as illustrated using GraphPad Prism 8.0.1. statistical software performed according to One-Way ANOVA (Tukey; HSD post hoc). A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Total phenol and flavonoid contents of extracts

The yields, total phenol, and flavonoid contents of *Helianthemum ledifolium* extracts are given in Table 1. Total phenol and flavonoid amounts of the extracts were determined spectrophotometrically. The methanol and water extracts of *H. ledifolium* varieties total phenol and flavonoid contents are as follows: var. *ledifolium* > var. *lasiocarpum* > var. *microcarpum* (Table 1).

Table 1. Total phenol, flavonoid contents, and yields of the extracts

Extracts of <i>H. ledifolium</i> varieties	Yield [%]	Total phenol [mg GAE per g extract]	Total flavonoid [mg QUE per g extract]
<i>Ledifolium</i> water extract (LedW)	11.6	167±1.282 ^a	8.36±0.64
<i>Ledifolium</i> methanol extract (LedM)	12.8	235.2±2.6	23.43±0.30
<i>Microcarpum</i> water extract (MicW)	5.05	93.29±0.87	1.58±0.27
<i>Microcarpum</i> methanol extract (MicM)	10.95	186.4±2.9	16.18±0.08
<i>Lasiocarpum</i> water extract (LasW)	7.43	124.6±2.81	2.36±0.25
<i>Lasiocarpum</i> methanol extract (LasM)	8.18	227.8±3.45	22.87±2.01

a: Mean ± SD

3.2. Antioxidant activity of extracts

The DPPH and ABTS radical scavenging activities of *H. ledifolium* varieties extracts are given in Figure 2. The results were satisfactory when compared with the activities of standard ascorbic acid. The extracts were evaluated for radical scavenging effects, and effects from highest to lowest were found to be Led, Las, and Mic, respectively.

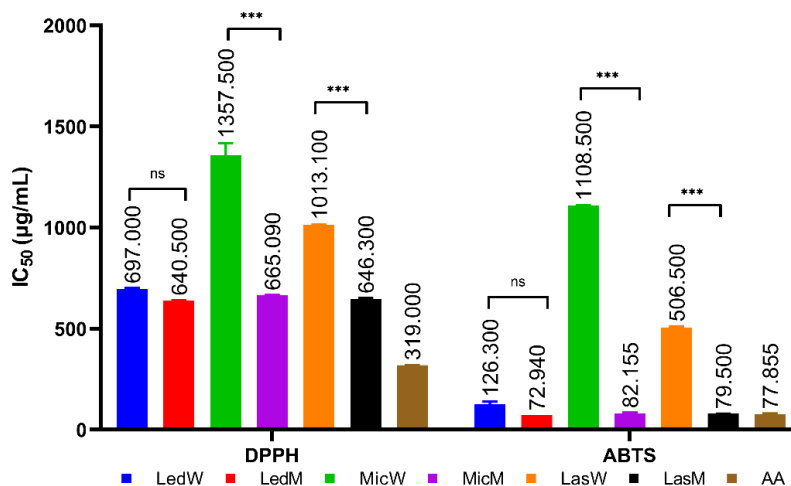


Figure 2. IC₅₀ values (µg/mL) of DPPH⁺ (A) and ABTS⁺ (B) radical scavenging activities for *H. ledifolium* varieties. ns: No significant difference, ****p*<0.05

3.3. Amoebicidal effect of extracts

The Two-Way ANOVA test was used to compare *H. ledifolium* varieties according to hours and doses. This can be interpreted as the survival rate of *A. castellanii* decreases as the exposure time increases. The results of pairwise comparisons for hours with significant differences are *p*< 0.05. The amoebicidal effects of *H. ledifolium* varieties extracts on *A. castellanii*, depending on time and concentration, are shown in Tables (2-4) and Figures (3-5).

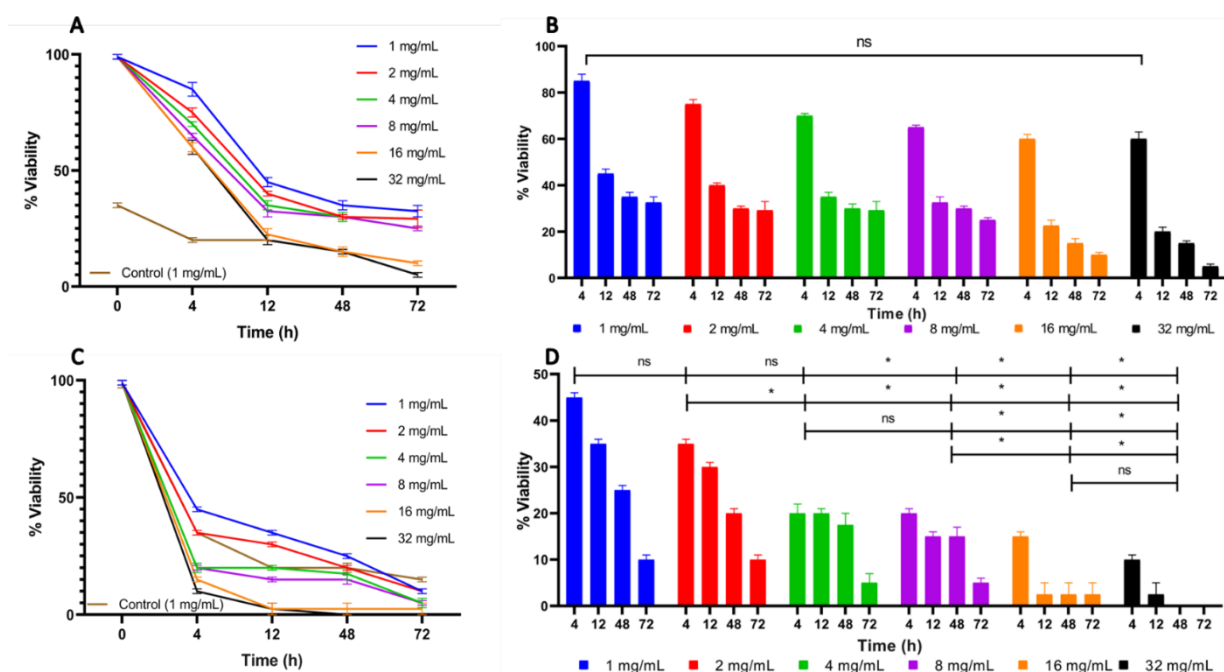


Figure 3. The effect of various concentrations of LedW (A, B) and LedM (C, D) on the cell viability of *A. castellanii* trophozoites at different times (hours). Control: fluconazole, ns: No significant difference, **p*<0.05

Table 2. LD₅₀, LD₉₀, LT₅₀, and LT₉₀ values (95% confidence limits) of *H. ledifolium* var. *ledifolium* extracts, estimated by probit analysis

Lethal Concentration Doses of LedW			Lethal Concentration Times of LedW		
Time	LD ₅₀ (95% confidence limit)	LD ₉₀ (95% confidence limit)	Doses	LT ₅₀ (95% confidence limit)	LT ₉₀ (95% confidence limit)
4 h	60.987 ^a (37.37-91.96)	271.05 (169.13-315.54)	1 mg/mL	16.716 ^b (4.54-23.506)	117.47 (80.414-211.18)
12h	0.6216 (0.255-1.0513)	258.88 (127.81-303.29)	2 mg/mL	11.602 (1.360-19.04)	94.592 (69.286-163.79)
48 h	0.188 (0.0721-0.897)	123.91 (89.061-140.135)	4 mg/mL	9.698 (0.468-17.51)	106.65 (72.718-173.353)
72 h	0.383 (0.00095-1.296)	21.853 (14.756-72.88)	8 mg/mL	8.157 (0.0137-16.877)	79.327 (49.339-99.903)
			16 mg/mL	5.189 (0.824-10.112)	52.465 (23.682-78.906)
			32 mg/mL	6.611 (0.796-19.165)	22.845 (13.757-32.462)
Lethal Concentration Doses of LedM			Lethal Concentration Times of LedM		
Time	LD ₅₀ (95% confidence limit)	LD ₉₀ (95% confidence limit)	Doses	LT ₅₀ (95% confidence limit)	LT ₉₀ (95% confidence limit)
4 h	1.107 ^a (1.060-3.073)	3.546 (2.640-5.428)	1 mg/mL	4.599 ^b (0.093-10.892)	58.956 (51.704-106.730)
12h	0.632 (0.097-1.350)	1.011 (0.000-2.745)	2 mg/mL	2.595 (0.006-8.023)	51.934 (47.331-84.694)
48 h	0.534 (0.134-1.059)	1.551 (0.365-2.942)	4 mg/mL	0.507 (0.246-4.307)	31.333 (0.345-36.144)
72 h	0.352 (0.070-1.614)	1.117 (0.036-2.927)	8 mg/mL	0.454 (0.098-3.550)	23.035 (1.191-30.282)
			16 mg/mL	2.152 (1.014-11.386)	7.245 (2.568-19.417)
			32 mg/mL	1.884 (0.286-4.414)	4.682 (1.235-8.549)

a: mg/ml; b: hours; -: not calculated.

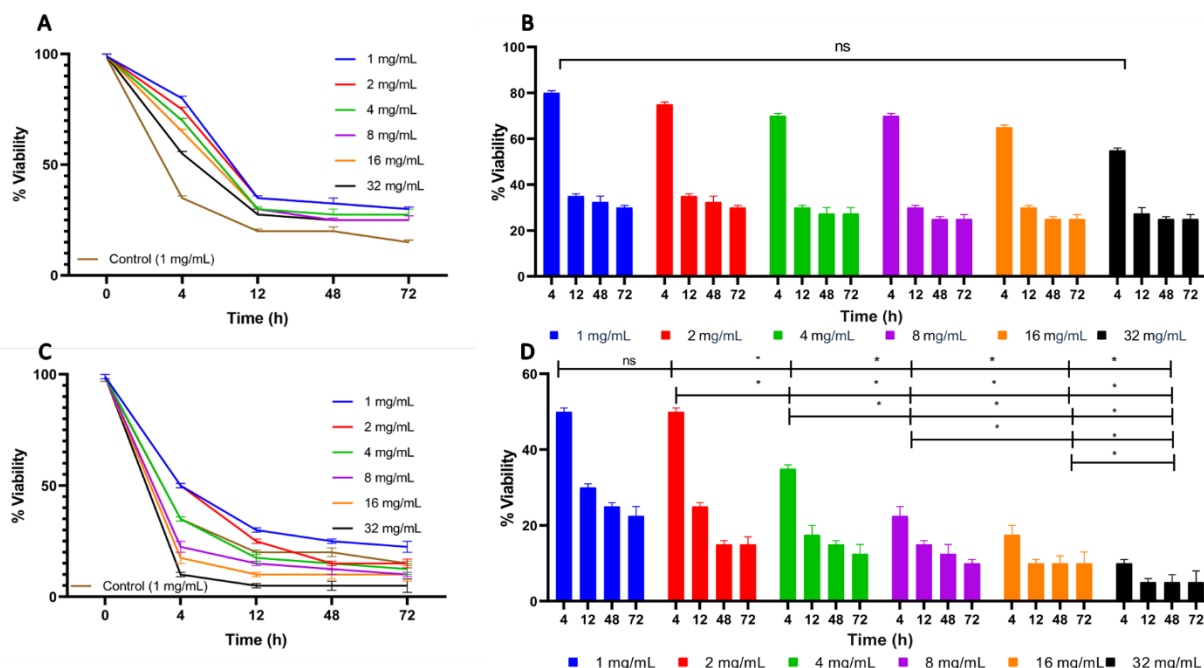
Figure 4. The effect of various concentrations of MicW (A, B) and MicM (C, D) on the cell viability of *A. castellanii* trophozoites at different times (hours). Control: fluconazole, ns: No significant difference, * $p < 0.05$

Table 3. LD₅₀, LD₉₀, LT₅₀, and LT₉₀ values (95% confidence limits) of *H. ledifolium* var. *microcarpum*, estimated by probit analysis

Lethal Concentration Doses of MicW			Lethal Concentration Times of MicW		
Time	LD ₅₀ (95% confidence limit)	LD ₉₀ (95% confidence limit)	Doses	LT ₅₀ (95% confidence limit)	LT ₉₀ (95% confidence limit)
4 h	92.701 ^a (34.613-149.265)	1.0074 (0.166-3.869)	1 mg/mL	14.629 ^b (0.328-23.262)	248.29 (145.95-313.93)
12h	0.0018 (0.000-0.0424)	2.442 (0.353-9.756)	2 mg/mL	12.011 (2.203-23.039)	154.58 (44.979-178.641)
48 h	0.00245 (0.000-0.0568)	0.522 (0.188-1.748)	4 mg/mL	9.047 (8.023-20.285)	140.21 (52.510-274.521)
72 h	0.0510 (0.005-0.398)	1.856 (0.273-2.118)	8 mg/mL	8.749 (1.446-18.368)	110.98 (84.112-170.215)
			16mg/mL	7.547 (0.00051-16.607)	102.08 (14.385-167.978)
			32mg/mL	7.161 (2.957-22.892)	30.72 (19.52-37.769)
Lethal Concentration Doses of MicM			Lethal Concentration Times of MicM		
Time	LD ₅₀ (95% confidence limit)	LD ₉₀ (95% confidence limit)	Doses	LT ₅₀ (95% confidence limit)	LT ₉₀ (95% confidence limit)
4 h	1.372 ^a (0.738-1.976)	35.202 (26.079-58.544)	1 mg/mL	3.552 ^b (0.246-8.267)	212.28 (121.74-252.12)
12h	0.2138 (0.071798-0.42298)	21.3314 (11.542-28.2145)	2 mg/mL	2.724 (0.444-5.942)	128.38 (93.436-168.64)
48 h	0.0447 (0.000631-0.225)	10.449 (9.675-12.799)	4 mg/mL	0.677 (0.0256-2.403)	86.689 (69.461-153.73)
72 h	0.0245 (0.00047-0.13069)	8.526 (6.788-10.417)	8 mg/mL	0.0476 (0.0016-0.279)	69.834 (61.185-92.1)
			16mg/mL	0.115 (0.598-2.305)	17.701 (6.738-27.561)
			32mg/mL	2.0414 (1.289-12.717)	7.404 (4.269-21.168)

a: mg/ml; b: hours; -: not calculated.

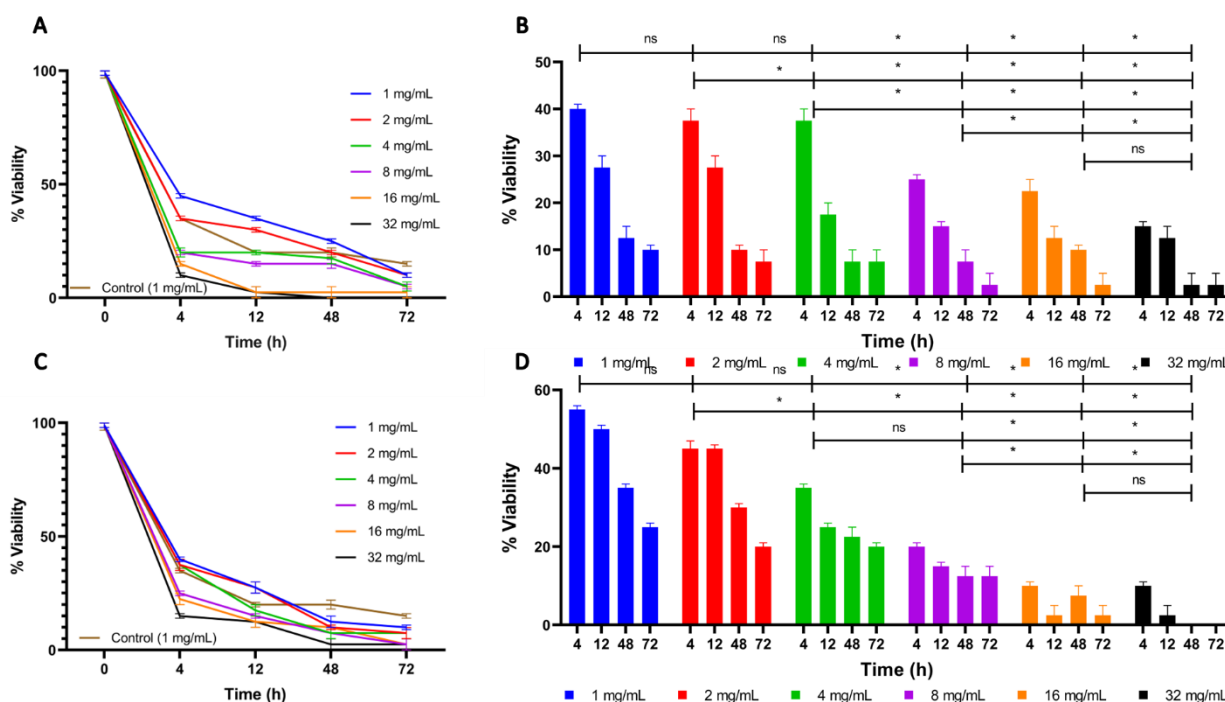


Figure 5. The effect of various concentrations of LasW (A, B) and LasM (C, D) on the cell viability of *A. castellanii* trophozoites at different times (hours). Control: fluconazole, ns: No significant difference, * $p < 0.05$

Table 4. LD₅₀, LD₉₀, LT₅₀, and LT₉₀ values (95% confidence limits) of *H. ledifolium* var. *lasiocarpum* extracts, estimated by probit analysis.

Lethal Concentration Doses of LasW			Lethal Concentration Times of LasW		
Time	LD ₅₀ (95% confidence limit)	LD ₉₀ (95% confidence limit)	Doses	LT ₅₀ (95% confidence limit)	LT ₉₀ (95% confidence limit)
4 h	1.397 ^a (0.737- 2.024)	23.84 (15.171- 35.171)	1 mg/mL	2.160 ^b (1.608-2.763)	68.492 (65.782-71.888)
12h	1.280 (0.511- 2.029)	9.3163 (4.951- 19.012)	2 mg/mL	4.977 (1.867-18.297)	20.239 (15.454-31.629)
48 h	1.407 (1.230- 3.466)	4.2978 (1.203- 5.97)	4 mg/mL	4.197 (1.988-17.329)	18.042 (11.352-30.377)
72 h	1.118 (1.499- 3.055)	3.5485 (1.256- 5.405)	8 mg/mL	3.102 (0.674-14.915)	13.815 (3.681-27.02)
			16 mg/mL	3.8229 (1.866-14.445)	11.148 (9.947 -23.277)
			32 mg/mL	3.0622 (0.787-12.664)	9.2988 (1.983-21.069)
Lethal Concentration Doses of LasM			Lethal Concentration Times of LasM		
Time	LD ₅₀ (95% confidence limit)	LD ₉₀ (95% confidence limit)	Doses		
4 h	0.497 ^a (0.057-1.161)	130.48 (56.279-210.63)	1 mg/mL	8.625 ^b (0.284- 16.409)	177.3 (98.92-219.7)
12h	0.042635 (0.000-0.234)	41.243 (24.384-83.97)	2 mg/mL	5.314 (0.001- 13.352)	138.2 (81.401-172.91)
48 h	0.0011031 (0.00-0.136)	2.388 (1.642-7.524)	4 mg/mL	1.335 (1.085- 7.046)	117.51 (71.635-156.25)
72 h	0.0034 (0.000-0.064)	0.996 (0.039-2.201)	8 mg/mL	0.143 (0.051- 2.935)	37.358 (29.8-63.11)
			16 mg/mL	1.795 (0.171 - 12.066)	6.678 (4.023- 20.302)
			32 mg/mL	1.884 (0.286- 4.414)	4.682 (1.235 - 8.549)

a: mg/ml; b: hours; -: not calculated.

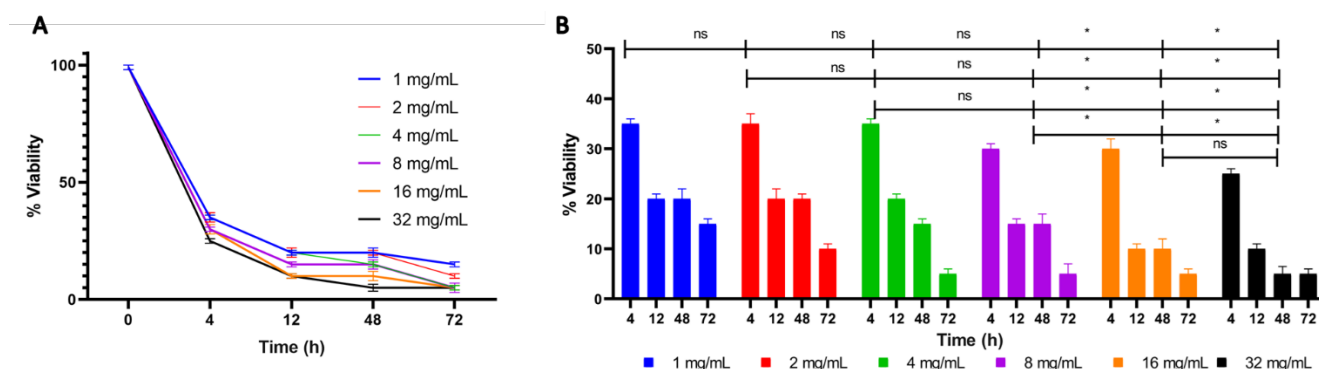


Figure 6. The effect of various concentrations of Control (fluconazole) on the cell viability of *A. castellanii* trophozoites at different times (hours). ns: No significant difference, **p*<0.05

Table 5. LD₅₀, LD₉₀, LT₅₀, and LT₉₀ values (95% confidence limits) of Fluconazole, estimated by probit analysis

Lethal Concentration Doses of LasW			Lethal Concentration Times of LasW		
Time	LD ₅₀ (95% confidence limit)	LD ₉₀ (95% confidence limit)	Doses	LT ₅₀ (95% confidence limit)	LT ₉₀ (95% confidence limit)
4 h	0.073708 (0.0018- 0.6302) _a	42.4 (4.889- 60.29)	1 mg/mL	1.022 (0.879 - 5.085) ^b	90.674 (65.108-108.7)
12h	0.0039 (0.000- 0.0402)	27.301 (18.92-69.406)	2 mg/mL	1.7557 (0.000 - 6.754)	47.21 (44.965-65.08)
48 h	0.049 (0.005- 0.367)	13.617 (11.132-34.915)	4 mg/mL	1.4647 (0.406 -6.218)	37.126 (25.166 -39.186)
72 h	0.018 (0.000- 0.159)	1.621 (0.236- 2.863)	8 mg/mL	3.656 (1.372 - 16.115)	15.693 (12.29 - 28.562)
			16mg/mL	3.153 (2.556 - 15.115)	14.006 (4.85 - 27.247)
			32mg/mL	3.3694 (1.714 - 13.325)	10.037 (6.440 - 21.908)

a: mg/ml; b: hours; -: not calculated.

4. Conclusions and discussion

The present study aimed to investigate the effects of different exposure and doses of *H. ledifolium* extracts in terms of parasite viability and to determine the amoebicidal effects. The present study suggests that the viability rate of the parasite is inversely proportional to the administered dose of the extract. However, this relationship depends on the specific dosage utilized. Additionally, the parasite's viability decreased depending on the period of exposure. The

overall results revealed that the extracts of *H. ledifolium* taxa effectively inhibited the *A. castellanii* compared with the control ($p < 0.05$).

In the literature, there have been total phenol, flavonoid contents, and antioxidant activity studies on different *Helianthemum* species. In the study by Rubio-Moraga et al. (2013), total phenolic content was expressed as mg gallic acid equivalents per liter (mg GAE/L) and varied markedly among 11 *Helianthemum* taxa, ranging from 50.72 ± 1.3 to 130.72 ± 3.1 mg GAE/L in water extracts and from 56.63 ± 2.0 to 138.45 ± 3.5 mg GAE/L in 80% methanolic extracts [12]. Baldemir et al. (2017) studied total phenol content of *H. canum* methanol extract was found to be 284.13 ± 0.30 , while total phenol content (gallic acid equivalents; mg/g) of the water extract was also found to be 244.55 ± 0.35 . Moreover, it was determined that the total flavonoid contents were 13.13 ± 0.10 and 14.01 ± 0.06 , respectively [21]. In another study, total phenol content (gallic acid equivalents; $\mu\text{g/g}$) of *H. sessilifolium* water extract was 7.35 ± 0.42 ; the total flavonoid content was calculated as 5.50 ± 0.19 [22]. Ibtissam et al. (2022) reported that the total phenol content (gallic acid equivalents; mg/g) of *H. lippii* water extract was found to be 183.12 ± 2.84 , and the total flavonoid amount was 72.00 ± 1.03 [23]. In another study, the total phenol content of the water extract of *H. lippii* aerial parts was reported as 134.67 (gallic acid equivalents; mg/g). There are also antioxidant effect studies on some *Helianthemum* species in the literature. For example, the IC_{50} value for DPPH radical scavenging activity of extract of *H. lippii* aerial parts was found as 27.79 ± 6.5 $\mu\text{g/ml}$ [24]. In the study by Rubio-Moraga et al. (2013), aerial parts water extracts from 11 *Helianthemum* taxa showed radical-scavenging activity, with IC_{50} values of 29.88 – 44.01 mg/mL (DPPH) and 18.70 – 108.65 mg/mL (ABTS). Methanol extracts displayed IC_{50} values of 25.30 – 66.20 mg/mL (DPPH) and 6.15 – 102.15 mg/mL (ABTS) [12]. Terfassi et al. (2022), IC_{50} values for DPPH and ABTS radical scavenging effects of methanol extract (80%) of *H. getulum* were reported as 21.25 ± 0.10 $\mu\text{g/ml}$ and 25.77 ± 0.52 $\mu\text{g/ml}$, respectively [25]. Our results were compared with the literature, showing that our findings were generally consistent with the phenolic contents and antioxidant activities of different *Helianthemum* species (Table 1 and Fig. 2).

Amoebicidal assays were conducted on *A. castellanii*, and the % viability (Fig. 3–5), LD_{50} , and LD_{90} values were determined (Tables 2–4). The results indicate that efficacy is positively correlated with both dosage and duration. Our studies indicate that all extracts examined have the potential to reduce the viability of *Acanthamoeba*, although the effects vary depending on the type of extract used (methanol and water). The data suggest that amoebicidal activity increases with increasing extract concentrations and exposure times. LasM extract exhibits a strong amoebicidal effect as early as 4 hours across the tested doses (Fig. 5), and a significant decrease in the rate of live parasites was observed. Exposure time analyses revealed that in the rapidly acting LedM, viability reached minimum levels within the first few hours, resulting in limited statistically significant differences in subsequent measurements (Fig. 3). In contrast, LasW and LasM showed more pronounced reductions after longer exposure times (48–72 hours) (Fig. 5).

The data clearly demonstrate the effects of different plant extracts on the viability of *Acanthamoeba* and highlight the role of the extraction solvent, extract concentration, and exposure time in this activity. In Fig. 3–5, water extracts (LedW, LasW, MicW) did not show statistically significant reductions in viability at lower concentrations (1 – 8 mg/mL) ($p > 0.05$). However, at higher concentrations (≥ 16 mg/mL), significant reductions in viability were observed. In contrast, methanol extracts (LedM, LasM, MicM) exhibited pronounced effects even at lower concentrations; LedM and MicM significantly reduced viability starting at 4 mg/mL (Fig. 3 and 4), while LasM showed a decrease from 8 mg/mL onward, with almost complete inhibition at higher doses (Fig. 5) ($p < 0.05$). Analyses of LD_{50} and LD_{90} values quantitatively confirmed the superior amoebicidal activity of methanol extracts compared to water extracts. Notably, for the Led group, the LD_{50} of LedM was approximately 55 times lower than that of LedW within the first 4 hours (Table 2), while MicM showed markedly higher efficacy compared to MicW initially (Table 3). LasM demonstrated a gradual but significant reduction in LD_{50} over time, reaching 0.0034 mg/mL at 72 hours, whereas LasW remained at 1.118 mg/mL (Table 4). In SI-Table 1, LasM (0.016 ± 0.04) and LedM (0.021 ± 0.04) at a concentration of 32 mg/mL showed the lowest viability rates, demonstrating that these extracts exhibit the strongest amoebicidal effects at the highest tested dose. Consequently, the highest toxicity potential was observed for LasM, which reached an LD_{50} value of 0.0034 mg/mL at 72 hours (Table 4), while the fastest killing profile among the tested extracts was observed for LedM at 32 mg/mL with $\text{LT}_{50} = 1.884$ hours and $\text{LT}_{90} = 4.682$ hours (Table 2). These findings indicate that methanolic extracts, in particular, are promising natural sources of bioactive compounds with rapid and potent amoebicidal activity.

In the analyses conducted to determine the killing rate, it was found that the effects of the extracts generally increased with dose, although LT estimates were not strictly monotonic across all concentrations due to variability reflected in the confidence limits. As shown in Table 2, the LT_{50} value of LedW at 1 mg/mL (16.716 hours) differed from those at higher concentrations, indicating the necessity of a threshold concentration for effective activity. When the time-dependent changes in viability rates presented in SI-Table 2 were examined, it was determined that the effects of all tested extracts on parasites increased with longer exposure times, leading to a gradual decrease in viability rates from 4 to 72 hours. The differences in efficacy among the extracts became particularly pronounced at 72 hours. While the viability rates were relatively high and similar at the 4-hour initial stage, the superiority of methanolic extracts became evident by 72 hours. At 72 hours, low viability rates were observed in the LedM extract (0.050 ± 0.04) and in the control group (0.050 ± 0.06), with similarly low values also recorded for LasW (0.042 ± 0.05) (SI-Table 2). The viability rates of LedM and LasM were lower than those of LedW (0.217 ± 0.11) and MicW (0.271 ± 0.02), which maintained the highest viability rates. These results demonstrate that the biological activity of methanolic extracts

becomes statistically more effective than that of water extracts over time. LedW and MicW extracts can thus be interpreted as the least effective among the tested extracts during the 72 hours.

The LasM extract achieved an LD₅₀ of 0.0034 mg/mL at 72 hours (Table 4), which is nearly 5 times lower than the LD₅₀ value obtained for the Fluconazole control at 72 hours (0.018 mg/mL) (Table 5). This difference is biologically significant. Furthermore, when analyzing the killing rate (LT₅₀), the MicM extract showed an LT₅₀ of 0.0476 hours (Table 3) at 8 mg/mL, demonstrating a much faster effect compared to the fastest LT₅₀ of the Fluconazole control (1.022 hours at 1 mg/mL) (Table 5). The lowest *Acanthamoeba* viability rate observed at 72 hours was 0.050 ± 0.04 (LedM), which was statistically comparable to the viability of the Fluconazole control at the same time point (SI Table 2) but was achieved by LedM at a higher lethal dose (LD₅₀ of 0.352 mg/mL at 72 h for LedM vs. LD₅₀ of 0.018 mg/mL for Fluconazole) (Tables 2 and 5). The highly favorable LD₅₀ and LT₅₀ values of LasM and MicM extracts confirm their substantial antiparasitic potential, indicating that these natural extracts are effective alternatives that may surpass the standard drug fluconazole in certain aspects of amoebicidal activity.

Chemical composition and antioxidant analyses further showed that methanol extracts were significantly richer in total phenols and flavonoids compared to water extracts, a finding supported by lower IC₅₀ values in DPPH and ABTS assays, indicating higher antioxidant activity. Overall, these results indicate that methanol extracts not only exhibit higher biological activity but also that their chemical composition underlies this efficacy.

Herein, the amoebicidal and antioxidant activities of *H. ledifolium* varieties are reported. Given the increasing resistance and treatment failures of existing drugs for the treatment of *Acanthamoeba* infections, our study highlights the potential for developing new therapeutic agents derived from natural sources. This pioneering study demonstrates that *H. ledifolium* taxa could serve as a valuable natural resource for future antiparasitic drug development. In light of these promising results, *in vitro* studies should be supplemented with *in vivo* models to elucidate the mechanism of action of the extracts at the molecular level and to assess their potential toxicity. Our paper represents an important step toward the development of new and safe antiparasitic products derived from nature.

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