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Investigation of *Sambucus ebulus* Plant Extract with Regard to its In-vitro DNA Protective Action, Cytotoxic Effect and Amoebicidal on *Acanthamoeba castellanii* Trophozoites

Bülent KAYNAK[1*], Gülizar AYDOĞDU[2], Zeynep KOLÖREN[3]

Abstract

The amoebicidal, DNA protective and cytotoxic activities of the ethanolic leaf extract of *Sambucus ebulus* against *Acanthamoeba castellanii* trophozoites were investigated. The extract at different concentrations (45, 22.5, 11.25, 5.62, 2.8, 1.4, 0.7 mg/mL) was found to amoebicidal effect on trophozoites. At a concentration of 45 mg/mL, the extract completely eliminated trophozoites by the 72nd hour. IC_{50} values were 1.9, 3.3, 4.8 mg/mL at 72, 48, 24 hours, respectively. Cytotoxicity in HeLa cells was assessed via 3-(4.5-dimethylthiazol-2 yl)-2.5-diphenyltetrazolium bromide (MTT) assay, revealing dose-dependent viability reduction with an IC_{50} of 1 mg/mL at 72 hours DNA-protective effects were observed against hydroxyl radical-induced damage in pBR322 plasmid DNA. Lower extract concentrations (5.62, 2.8, 1.4 mg/ml) effectively inhibited DNA damage, while higher concentrations (45, 22.5, 11.25 mg/ml) showed weaker effects. Phytochemical analysis using Gas Chromatography-Mass Spectrometry (GC-MS) identified the bioactive compounds responsible for these effects. In conclusion, the potent amoebicidal activity, DNA protective properties, and controlled cytotoxicity of *Sambucus ebulus* extract suggest its potential as a therapeutic agent not only for antiparasitic treatments but also for the prevention of DNA damage-related diseases. The findings indicate that the extract could be considered as an alternative or complementary approach for preserving genomic integrity and for clinical applications.

Keywords: *Acanthamoeba castellanii*, GC-MS, MTT, pBR322 plasmid DNA, *Sambucus ebulus*.

Sambucus ebulus Bitki Özütünün İn-vitro DNA Koruyucu Etkisinin, Sitotoksik Etkisinin ve *Acanthamoeba castellanii* Trofozoitleri Üzerinde Amoebisidal Etkisinin Araştırılması

Öz

Sambucus ebulus bitkisinin etanollü yaprak özütünün *Acanthamoeba castellanii* trofozoitlerine karşı amoebisidal, DNA koruyucu ve sitotoksik etkileri araştırılmıştır. Çalışmada, farklı konsantrasyonlarda (45, 22.5, 11.25, 5.62, 2.8, 1.4, 0.7 mg/mL) uygulanan özütün trofozoitler üzerinde belirgin amoebisidal etki gösterdiği saptanmıştır. Özellikle 45 mg/mL konsantrasyonda, özüt 72. saatte trofozoitlerin tamamen elimine edilmesini sağlamıştır. IC_{50} değerleri 72, 48, 24. saatlerde sırasıyla 1.9, 3.3, 4.8 mg/mL olarak hesaplanmıştır. Özütün HeLa hücrelerindeki sitotoksik etkisi, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) testi ile değerlendirilmiş ve doza bağlı bir canlılık azalması gözlemlenmiştir. Bu analiz sonucunda, 72. saatte IC_{50} değeri 1 mg/mL olarak belirlenmiştir. Ayrıca, özütün pBR322 plazmid DNA üzerinde hidroksil radikalinin neden olduğu DNA hasarını önleme kapasitesi incelenmiştir. Düşük konsantrasyonlardaki (5.62, 2.8, 1.4 mg/mL) özütün DNA hasarını etkili bir şekilde inhibe ettiği, yüksek konsantrasyonlardaki (45, 22.5, 11.25 mg/mL) etkilerin ise daha zayıf olduğu gözlenmiştir. Fitokimyasal analiz, Gaz Kromatografisi-Kütle Spektrometresi (GC-MS) yöntemiyle yapılmış ve etkilerden sorumlu biyoaktif bileşikler tanımlanmıştır. Sonuç olarak, *S. ebulus* özütünün güçlü amoebisidal etkisi, DNA koruyucu özellikleri ve kontrollü sitotoksisitesi, bu özütün sadece antiparaziter tedavilerde değil, aynı zamanda DNA hasarına bağlı hastalıkların önlenmesinde de potansiyel bir tedavi seçeneği olabileceğini göstermektedir. Elde edilen bulgular, özütün hem genomik bütünlüğün korunmasında hem de klinik uygulamalarda alternatif veya tamamlayıcı bir yaklaşım olarak değerlendirilebileceğini işaret etmektedir.

Anahtar Kelimeler: *Acanthamoeba castellanii*, GC-MS, MTT, pBR322 plazmid DNA, *Sambucus ebulus*.

[1,2,3]Ordu University, Molecular Biology and Genetics Department, Ordu, Türkiye, bulentkaynak2004@gmail.com gulizaraydogdu@yahoo.com zeynep.koloren@gmail.com

[*]Sorumlu Yazar/Corresponding Author

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1. Introduction

Although parasitic diseases are a widespread health issue, they have often been neglected, leading to significant economic damage, especially in endemic regions (Leder and Weller, 2003; Kaynak et al., 2019). *Acanthamoeba* spp., one of the free-living amoebae, is a heterotrophic and eukaryotic protozoan that lives freely in soil and water (Aykur and Dagci, 2022). *Acanthamoeba* spp. are in different environments. *Acanthamoeba* species can be found in swimming pools, packaged and distilled water, seawater, stagnant water, lakes, river water, ventilation ducts, sewage, ocean sediment, beaches, surgical instruments, contact lenses, hospitals, dialysis units, and various human tissues (Khan, 2006; Ceniklioglu and Duzlu, 2022). *Acanthamoeba* has two life forms: trophozoite, which is active, and cyst, which is resistant to harsh conditions (Saygi and Polat, 2003; Kaynak et al., 2019; De Lacerda and Lira, 2021).

Acanthamoeba species are transmitted to humans through breathing, eyes, and skin. *Acanthamoeba* keratitis (AK), which is caused by *Acanthamoeba* species; is a painful corneal disease, worsens and threatens visual function, and even causes corneal ulceration and blindness if left untreated. Granulomatous amoebic encephalitis (GAE) is a rare and often fatal disease, caused by some *Acanthamoeba* species, chronic and with a poor prognosis, characterized by a Central Nervous System (CNS) infection that also affects the lungs in children and adults with strong or weak immune systems. It is an infectious disease that can also be seen in adults. Cutaneous acanthamoebiasis is a disease characterized by hard erythematous nodules and skin ulcers, which is mostly seen with CNS involvement in AIDS patients (Marciano-Cabral and Cabral, 2003; Kaynak et al., 2019). The most effective way to combat these diseases is effective and successful treatment. However, the lack of development of antiparasitic vaccines, the limited availability of existing antiparasitic drugs, and the development of resistance by microorganisms against these drugs make the effective fight against parasitic diseases even more difficult. On the other hand, effective drug development efforts remain inadequate, especially in developing countries. However, the eukaryotic structure of parasites hurts the activity and selectivity of the developed drugs, reducing their effectiveness (Leder and Weller, 2003; Kaynak et al., 2019).

Sambucus ebulus is a rhizomatous herbaceous perennial plant belonging to the Caprifoliaceae family, growing in groups, erect, usually unbranched stems up to 1-2 m in height. *S. ebulus* is distributed in Southern and Central Europe, Northwest Africa and Southwest Asia (particularly Northern Iran) (Jabbari et al., 2017). In Turkey, it is distributed in the Mediterranean, Marmara, Black Sea, Central and Eastern Anatolia regions (Anonymous, 2024). *S. ebulus* has been found to have important nutritional value as it contains sugar, fiber, vitamins and minerals (Vlachojannis et al., 2010; Jabbari et al., 2017). The phytochemical composition of the plant has been reported as

flavanoids, anthocyanins, steroids, tannins, glycosides, cardiac glycosides, caffeic acid derivatives, vitamin C, chlorogenic acid, volatiles, ursolic acid, and phenols (Ebrahimzadeh et al., 2008; Schwaiger et al., 2011; Tasinov et al., 2013). In addition, the plant showed to store other chemical compounds such as phytosterols, triterpenes, iridoid glycosides and lectins (Schwaiger et al., 2011; Yesilada et al., 2014; Jabbari et al., 2017). Essential polyunsaturated fatty acids (a-linolenic, linoleic acid, oleic acid and palmitic acid) are other components of *S. ebulus* seeds (Jabbari et al., 2017

S. ebulus fruits have been used as food and medicine in Europe for thousands of years. In traditional medicine, different parts of the plant used for treating variying ailments such as rheumatoid arthritis, fever, certain infections, bites and sore throat. In traditional Persian medicine, it is used as a painkiller in painful conditions such as joint pain, fractures and dislocations, and various bone and joint disorders. The plant is also used in the treatment of uterine diseases, burns, gout, dental disorders, dehydration, fistula, and as a diuretic (Jabbari et al., 2017). The plant is also used to treat arthritis, sore throat, and bee stings. In Anatolia, the leaves of the plant have been used externally for abscesses, wounds, sunstroke, snake bites, edema, colds, eczema, rheumatic pain, and high fever. In addition, the leaves are laxative, diuretic, and diaphoretic and have been used against hemorrhoids and stomach pains (Yesilada et al., 2014; Jabbari et al., 2017).

In a previous study, the antiviral properties of *Sambucus nigra* (SN) and *Sambucus ebulus* (SE) extracts were evaluated against herpes simplex virus type-2 (HSV-2). The analysis revealed that these extracts contain phenolic acids, flavonoids, and anthocyanins, with rutin and chlorogenic acid being predominant in the flower and leaf extracts, while anthocyanins were more abundant in the fruit extracts. The highest antioxidant activity was observed in SN fruit extract (Seymenska et al., 2023). Recent studies have demonstrated the significant immunomodulatory and anti-inflammatory potential of *Sambucus ebulus* L. (SE) fruits, primarily due to their rich phytochemical composition. The aqueous extract of SE fruits (FAE) was found to contain a range of bioactive compounds, including hydroxycinnamic acids, proanthocyanidins, and anthocyanins, alongside newly reported amino acids, organic acids, alcohols, fatty acids, and esters. These compounds contribute to the extract's ability to modulate immune responses and inflammation (Tasinov et al., 2021). A Previous research has demonstrated the potent antiparasitic activity of silver nanoparticles derived from *Sambucus ebulus* (*Ag-NPs-S. ebulus*). This study highlighted the significant effectiveness of these nanoparticles in inhibiting the proliferation of *Toxoplasma gondii* both in vitro and in vivo, with a stronger parasiticidal effect compared to traditional treatments. This supports the potential of *S. ebulus* nanoparticles as a promising antiparasitic treatment option (Hematizadeh et al., 2023).

The aim of the presented study was to investigate the DNA protective effect, cytotoxic effect and amoebicidal effect of ethanolic extract obtained from *S. ebulus* (black elderberry) leaves against *A. castellanii* trophozoites.

2. Materials and Methods

2.1. Preparation of Plant Leaf Extract

The plant was collected from hazelnut gardens close to Ordu University Cumhuriyet Campus. The leaves of the plant were washed with water and dried with blotting papers. Weighed 100 g on a precision balance and combined with 300 mL ethanol in a grinder. The mixture was then put in a sterile payrex bottle and kept in a shaker at 150 rpm, 40 % humidity, 20 °C for 72 hours. After 72 hours, filter paper was used for filtration and then ethanol was removed in an evaporator at 40 °C. Bacteria were isolated by passing through a vacuum filter with bacterial filter (pore diameter: 0.22 µm). The final concentration of the extract was adjusted as 45 mg/mL (Kaynak et al., 2019).

2.2. *Escherichia coli* Culture

E. coli (ATCC 25922) was inoculated into EMB (Eosin Methylene Blue) medium by sterile inoculum and incubated in an oven at 36 °C for 1 day, after which the colonies giving yellow-green metallic reflex were washed with Ringer broth and collected into sterile ependorf tubes.

2.3. *A. castellanii* Trophozoite Culture

Pre-prepared 1 mL of *E. coli* (ATCC 25922) suspension was added to the Ringer agar medium and then 300 µL of *A. castellanii* (ATCC 30010) strain was added to the same medium and inoculated with a sterile core. After incubation in an oven at 26 °C for 96 hours, trophozoites were washed with Ringer Broth medium, collected and tested for the number of trophozoites per mL and viability with 0.4 % trypan blue stain on a thoma slide. The experiment started with $2x10^6$/mL trophozoite count and 100 % viability (Kaynak et al., 2019).

2.4. Testing the Amoebicidal Activity of a Plant Extract

Plant extracts prepared at different concentrations (45, 22.5, 11.25, 5.62, 2.8, 1.4 mg/mL) were placed in ependorf tubes as 200 µL and 200 µL of *A. castellanii* trophozoite was put to each ependorf tube. Negative control tube (trophozoite + sterile distilled water) and positive control tube (trophozoite + 0.05 % Chlorhexidine gluconate) were also prepared and kept in the oven at 26 °C for 24, 48, 72 hours and at the end of each period, 25 µL of 0.4 % trypan blue was added to 25 µL of parasite + plant extract mixture, and incubate at room temperature for 5 minutes and dead and live

trophozoites were counted separately on thoma slides. Each count was repeated three times (Malatyali et al., 2011; Kaynak et al., 2019).

2.5. MTT Cytotoxicity Assay

The cytotoxic effect of different concentrations (45, 22.5, 11.25, 5.62, 2.81, 1.40, 0.70 mg/mL) of leaf extract of *S. ebulus* on mammalian cells was tested using HeLa (Human cervix adenocarcinoma cell-ATCC) cell line. HeLa cells were replicated in high glucose DMEM (Dulbecco's Modified Eagle Medium) medium including 10 % FBS (Fetal Bovine Serum) and 2 mM L-glutamine in T75 cell dishes in an incubator containing 5 % CO_2 at 37 °C. The proliferating cells were passaged by trypsin. The HeLa cells removed by trypsin and counted on a thoma slide were seeded in DMEM (Dulbecco's Modified Eagle Medium) medium containing 10 % FBS (Fetal Bovine Serum) in transparent flat bottomed 96 well cell culture dishes with 10^4 cells per well. The next day, the cells were subjected to varying concentrations of leaf extract (45, 22.5, 11.25, 5.62, 2.81, 1.40, 0.70 mg/mL). Cells were subjected to plant leaf extract for 72 hours. Elabscience MTT Cell Proliferation and Cytotoxicity Kit was performed to test cytotoxicity at the end of the treatment. Medium was removed from the wells according to the kit protocol and 50 µL of serum-free DMEM and 50 µL of MTT reagent was put into per well. For background control, 50 µL of MTT standard was put to a well containing media only (no cells). The 96-well cell dish was incubated at 5 % CO_2, 37 °C for 3 hours. Then 150 µL of MTT standard was put into the wells. The 96 well cell culture dish was wrapped with a foil and shaken on an orbital shaker for 15 minutes. After 15 minutes, the absorbance was read at 570nm on a Tecan i-control™ Microplate Reader.

2.6. DNA Protective Activity of *S. ebulus* Extract on Hydroxyl Radical Induced DNA Damage

To investigate the effect of ethanol leaf extract of *S. ebulus* against hydroxyl radical (-OH) induced DNA damage, the protocol of Col Ayvaz et al., (2018) was modified and used. To demonstrate the protective efficacy of the ethanol extract from the plant sample against hydroxyl radical-mediated DNA damage, a reaction mixture was carefully prepared. This mixture, with a final volume of 15 µL, included 0.25 µg/µl pBR322 plasmid DNA, 2.5 mM Na2-EDTA, 2.5 mM Fe2SO4, 2% H2O2, and varying quantities of S. ebulus extract (ranging from 45, 22.5, 11.25, 5.62, 2.8, and 1.4 mg/mL) in 50 mM phosphate buffer (pH 7.0). The reaction mixture, containing different amounts of plant samples, was then incubated at 37 °C for 30 minutes. Following this, the samples were subjected to electrophoresis on 1% agarose (containing 0.5 µg/mL of ethidium bromide), and a

photograph was captured under UV light at the conclusion of the electrophoresis process in a running buffer (1x TAE).

2.7. Gas Chromatography Mass Spectrometry (GC-MS) Analysis of *S. ebulus* Extract

S. ebulus leaf extract was prepared at a concentration of 2000 ppm using DMSO (Dimethyl sulfoxide) solvent after the ethanol and water in the *S. ebulus* leaf extract were completely evaporated in the evaporator and GC-MS analysis was done on a Restek-Rtx-5 capillary column (30.0 m x 0.25 mm x 0.25 μm) using electron pulse mode at 70 ev; helium (99 %). A carrier gas at a constant flow rate of 1 mL/min and injection volume of 1 μL was used (split ratio 1/10) injector temperature 240 ºC, ion source temperature 200 ºC. The oven temperature started at 40 ºC for 2 min with a 4 ºC/min increase to 240 ºC (10 min) for a total run time of 55 min. El-Beltagi et al., (2019) was used with modification. The evaluation of the results made use of the Library of Natural and Synthetic Compounds of Flavors and Fragrances (FFNSC3), the National Institute of Standards and Technology (NIST) and a specialized mass spectral database (WILEY).

2.8. Statistical Analysis

The cellular changes in the control group (CG) to which *S. ebulus* extracts were not added and the test groups to which 6 different concentrations of *S. ebulus* extracts were added were analyzed comparatively. Data entry for the changes at 24, 48 and 72 hours of the test groups containing *S. ebulus* extracts and the cytotoxicity test of different concentrations of *S. ebulus* extracts on mammalian cells (HeLa) were made with Microsoft Excel and IBM SPSS Statistics 27.0.1 programs and descriptive data analysis and graphics were performed with IBM SPSS Statistics 27.0.1 package program. Data are expressed as mean (Mean) ± standard error (SD). In IBM SPSS Statistics 27.0.1 program, the "p" value (probability) was compared by accepting 1 % error. Confidence intervals were determined with 99 % probability. The results of % viability counts showing the amoebicidal activity of the extracts were evaluated by Post-Hock multiple comparison analysis and Tukey pairwise group comparison with IBM SPSS Statistics 27.0.1. Logarithmic regression analysis was used to calculate the 50 % inhibitory (IC_{50}) values of different concentrations of plant extracts with lethal effect on trophozoites and HeLa cells. The values of different concentrations of extracts providing 50 % cell death were calculated by logarithmic regression graph. The % cell death values of the amoebicidal effect of *S. ebulus* extract concentrations at different times in the experiments compared to control cells were calculated. Inhibitor concentrations (50 %) were made in three replicate experiments. In

addition, explanatory variables were visualized using Principal Component Analysis (PCA) Jamovi 2.4.11 to summarize the results of all concentrations.

3. Findings and Discussion

3.1. Amoebicidal Activity of *S. ebulus* Extract on *A. castellanii* Trophozoites

The extract of *S. ebulus* had a lethal effect on most of the trophozoites at the end of the 24th and 48th hours at a concentration of 45 mg/mL. At the same concentration, no viable trophozoites were found at the 72nd hour. It was determined that the concentration of 22.5 mg/mL had a significant lethal effect at the 72nd hour (Figure 1).

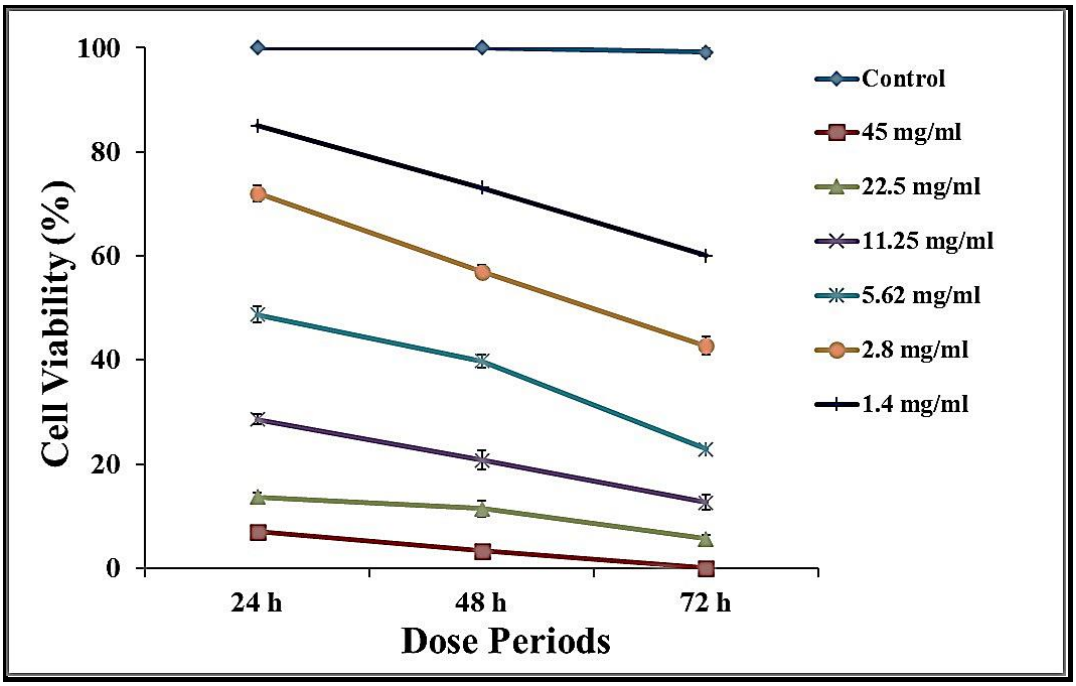

Figure 1. The effect of different concentrations of *S. ebulus* extract on the cell viability of *A. castellanii* trophozoites at varying times (hours)

The IC_{50} values of the ethanolic leaf extract of *S. ebulus* on *A. castellanii* trophozoites were determined as 1.9, 3.3 and 4.8 mg/mL at the 72nd, 48th and 24th hours, respectively (Table 1).

Table 1. IC_{50} value of *S.ebulus* ethanolic extract on *A. castellanii* trophozoites at different concentrations

The stage of *A. castellanii*	Experimental period	50%inhibitor concentration (IC_{50})
Trophozoites	72nd h	1.9 mg/mL
	48th h	3.3 mg/mL
	24th h	4.8 mg/mL

When the effects of the extract of *S. ebulus* on the viability of *A. castellanii* trophozoites were investigated, it was shown that the viability rates decreased as the extract dose increased. The highest viability was measured at the lowest dose of 1.4 mg/mL and the lowest viability was measured at the highest dose of 45 mg/mL. When the effects of application times on viability were examined, it was determined that the viability rates decreased as the waiting time increased. The highest viability was measured at the end of 24th hour, while the lowest viability was measured at the end of 72nd hour.

In addition, when the extract at 45 mg/mL concentration was compared among itself at three hours, The cell viability at 48 hours was not significantly different from that at 72 hours while a statistical difference was found between both hours and 24 hours. When the extract at 22.5 mg/mL concentration was compared among itself in each three hours, significant statistical differences were found between % cell viability at 24, 48, 72 hours. When the extract at 11.25 mg/mL concentration was compared among itself in each of the three hours, there was no significant statistical difference between the % cell viability at 24 and 48 hours, while a statistical difference was found in both hours compared to 72 hours. When the extracts at concentrations of 5.62, 2.8, 1.4 mg/mL were compared among themselves in each of the three hours, the % cell viability data at 24, 48 and 72 hours showed statistically significant variations at all concentrations (Table 2).

Table 2. Effect of *S. ebulus* ethanolic leaf extract on cell viability on *A. castellanii* trophozoites at various doses and times

Concentration/Hour	24th h	48th h	72nd h	Σ
Control	100 ± 0.00^{a}	100 ± 0.00^{a}	99.16 ± 1.44^{a}	99.22 ± 0.83^{a}
1.4 mg/mL	85.01 ± 2.31^{b}	73.17 ± 2.16^{c}	60.02 ± 2.16^{d}	72.73 ± 11.05^{b}
2.8 mg/mL	72.06 ± 2.67^{c}	56.99 ± 2.16^{d}	42.72 ± 2.98^{ef}	57.25 ± 12.90^{c}
5.62 mg/mL	48.76 ± 2.38^{e}	39.79 ± 2.75^{f}	22.89 ± 2.14^{gh}	37.14 ± 11.56^{d}
11.25 mg/mL	28.62 ± 1.78^{g}	20.77 ± 3.17^{gh}	12.65 ± 2.59^{ij}	20.68 ± 7.26^{e}
22.5 mg/mL	15.07 ± 2.50^{hi}	11.46 ± 2.74^{ij}	5.65 ± 1.14^{jk}	10.72 ± 4.55^{f}
45 mg/mL	7.04 ± 1.88^{jk}	3.37 ± 1.41^{k}	0.00 ± 0.00^{k}	3.47 ± 3.27^{g}
Σ	50.93 ± 34.09^{a}	43.65 ± 33.41^{b}	34.73 ± 33.66^{c}	

There is a statistical difference between the values indicated with different letters such as a,b,.., k.

To summarize the % viability results of all extract concentrations on *A. castellanii* trophozoites, the explanatory variables were visualized by PCA. Separating the right and left side of the axis with an explanation rate (99.93 %), the doses at 45, 22.5, 11.25 and 5.62 mg/mL are negatively correlated with all other doses (2.8 and 1.4 mg/mL), including the control (Figure 2).

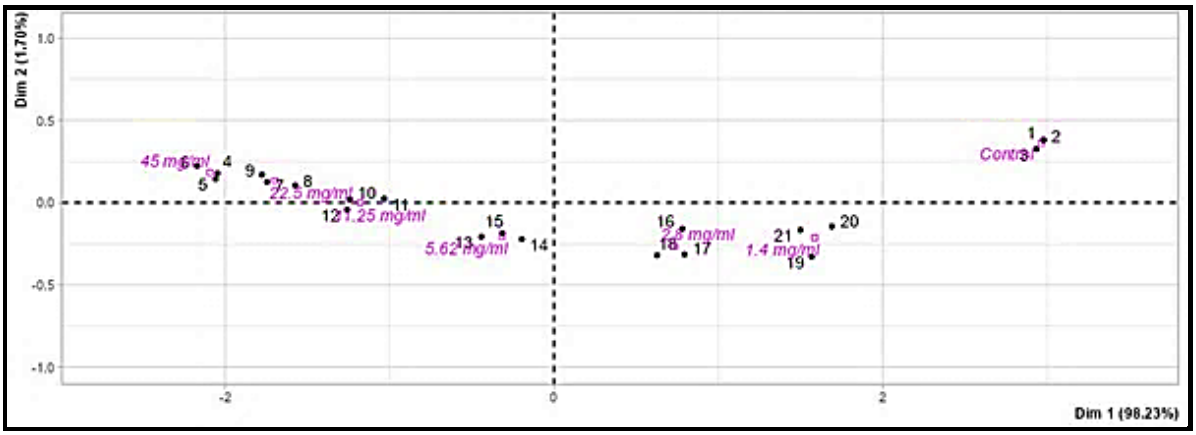

Figure 2. PCA representation of *S. ebulus* extract at different concentrations

The effect of different concentrations of *S. ebulus* ethanolic extract on % viability of active components at different times is presented (Figure 3). The % cell viability values of *S. ebulus* at 24 hours for all applied doses show a significant negative correlation with the values at 72 hours. The 48th hour cell viability data is located in the middle of the axis and it was observed that cell viability decreased in the time period after this time. In this context, 48th hour can be considered as the threshold value for all doses. When the eigenvalue and cumulative variance percentage of *S. ebulus* extract were analyzed, all axes explained 99.9 % of the variances (Table 3).

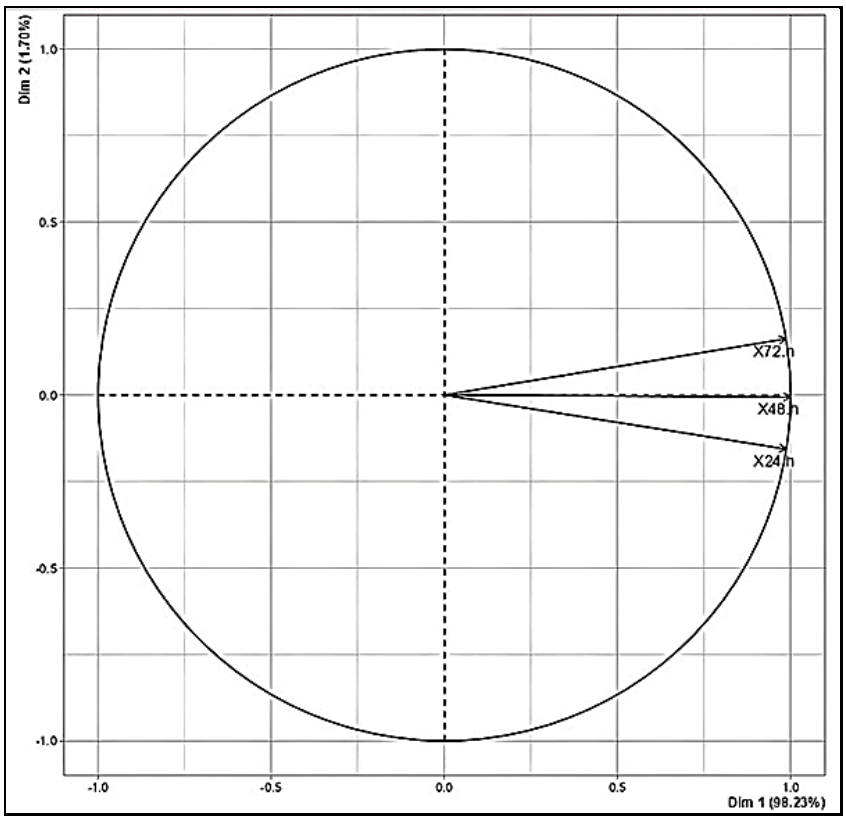

Figure 3. Illustration of the active components of the ethanolic leaf extract of *S. ebulus* at various doses and times

Table 3. Eigenvalue and Cumulative Variance Percentage of *S. ebulus* extract

	Eigenvalue	% of the variance	Cumulative %
Dim. 1	2.94697	98.2324	98.2
Dim. 2	0.05089	1.6965	99.9

3.2. Dose-dependent Cytotoxic Effect of *S. ebulus* Extract in HeLa Cells

Ethanolic leaf extract of *S. ebulus* at different concentrations (45, 22.5, 11.25, 5.62, 2.8, 1.4, 0.7 mg/mL) was incubated in HeLa cell line for 72 hours. At 72 hours, the IC_{50} value was 1 mg/mL. It is observed that the extract decreased cell viability depending on the dose. However, at the end of 72 hours, it was found that doses below 1 mg/mL did not show cytotoxic effect for half of the cells (Figure 4).

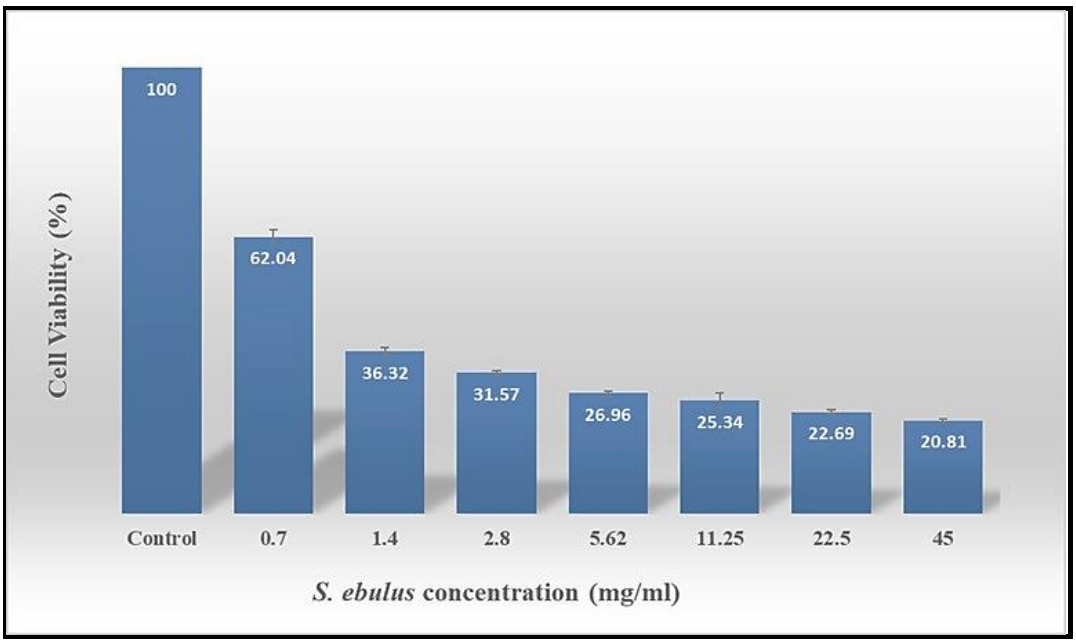

Figure 4. Cytotoxicity of ethanolic leaf extract of *S. ebulus* at different concentrations on HeLa cells at 72 hours.

3.3. DNA Protective Activity of *S. ebulus* Extract

In order to investigate the inhibitory effect of *S. ebulus* ethanolic leaf extract on (-OH) induced DNA damage, the repair of free radical-induced pBR322 plasmid DNA breaks was examined by agarose gel electrophoresis. pBR322 plasmid DNA exists in super coiled state under non-stress conditions. With DNA damage induced by hydroxyl radical, DNA becomes circular and linear. When DNA damage is prevented, as in the Trolox positive control, the DNA returns to the supercoiled state. It was found that high concentrations of the extract (45, 22.5 and 11.25 mg/mL) had weak effects on the inhibition of (-OH) induced DNA damage, but low concentrations of the extract (5.62, 2.8 and 1.4 mg/mL) had strong effects on the inhibition of (-OH) induced DNA damage (Figure 5).

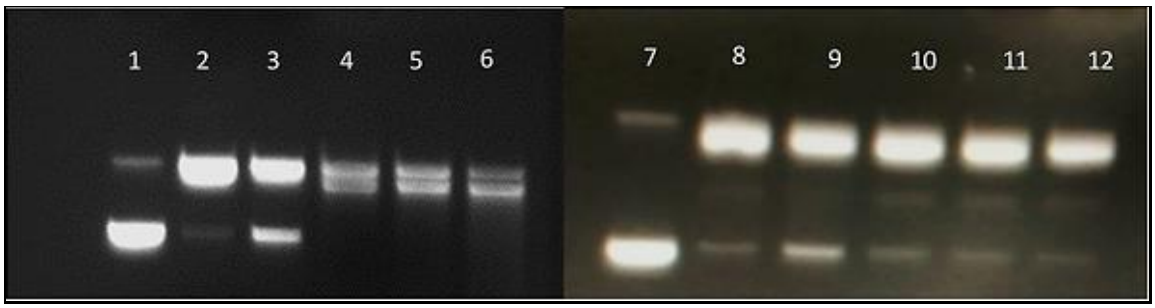

Figure 5. Agarose gel electrophoresis image showing DNA protection activity of *S. ebulus* samples on plasmid DNA damage caused by hydroxyl radical.

Lane 1and 7: pBR322 DNA;
Lane 2 and 8: pBR322 DNA+ Na_2EDTA+H_2O_2+ Fe_2SO_4,
Lane 3 and 9: pBR322 DNA + Na_2EDTA +H_2O_2+ Fe_2SO_4 + Trolox,
Lane 4 to 6: 45, 22.5, 11.25 mg/mL; Lane 10 to12: 5.62, 2.8, 1.4 mg/mL different concentrations of *S. ebulus* extract used as a substitute for Trolox.

3.4. Chemical Composition of *S. ebulus* Extract by GC-MS Analysis

When the ethanolic leaf extract of *S. ebulus* was analyzed by GC-MS (Table 4), it was found that some compounds (Dimethylsulfoxonium formylmethylide and 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one) and plants containing these compounds have antiparasitic activities as a result of literature review. Shibula and Velavan, (2015) determined the presence of various compounds such as 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one in GC-MS analysis of methanolic extract of *Annona muricata* leaves. Miranda et al., (2021) emphasized that ethanolic leaf extracts of *A. muricata* showed in vitro anti-toxoplasmal activity and can be considered as a complementary and/or alternative drug in the treatment of parasitic diseases such as toxoplasmosis. In addition, Jha et al. (2022) determined the components in the essential oil of *Jasminum sambac* by GC-MS analysis. They determined antibacterial, antioxidant, antifungal and antimalarial (even at low concentrations) activity of the oil. In the GC-MS analysis of the oil, the main compound was Dimethylsulfoxonium formylmethylide (85.33 %)

Table 4. Phytocompounds found in *S. ebulus* leaves by GC-MS analysis

Peak	Ret. Time	Area %	Name	CAS	Formula	Molecular Weight (MW)	Library
1	10.062	8.76	Methane, sulfinylbis- (CAS) Dimethyl sulfoxide	67-68-5	C_2H_6OS	78	WILEY
2	10.475	3.15	Methane, sulfonylbis- (CAS) Dimethyl sulfone	67-71-0	$C_2H_6O_2S$	94	WILEY
3	26.271	4.3	2-Amıno-9-(3,4-Dıhydroxy-5-Hydroxymethyl-Tetrahydro-Furan-2-Yl)-3,9-Dıhydro-Purı	0-00-0	$C_{10}H_{13}N_5O_5$	283	WILEY
4	30.894	4.92	1,2,4-Cyclopentanetriol, (1.alpha.,2.beta.,4.alpha.)- (CAS)	42142-32-5	$C_5H_{10}O_3$	118	WILEY
5	31.433	24.4	Quınıc Acıd	77-95-2	$C_7H_{12}O_6$	192	WILEY
6	31.785	2.07	2-Deoxy-D-ribose	533-67-5	$C_5H_{10}O_4$	134	WILEY
7	34.294	10.38	Alpha.-L-Rhamnofuranose, Tetra-O-Acetyl-	0-00-0	$C_{14}H_{20}O_9$	332	WILEY
8	51.743	7.52	3-(6,6-Dımethyl-5-Oxo-Hept-2-Enyl)-Cyclohexanone	83040-95-3	$C_{15}H_{24}O_2$	236	WILEY
9	51.895	5.56	Myrtensaeure, Butylester	62486-91-3	$C_{14}H_{22}O_2$	222	WILEY
10	52.941	7.8	Pentanoyl chloride (CAS) Valeryl chloride	638-29-9	C_5H_9CLO	120	WILEY
11	10.062	8.76	Sclerosol	67-68-5	C_2H_6OS	78	FFNSC3
12	10.475	3.15	Norborneol <exo-2->	497-37-0	$C_7H_{12}O$	112	FFNSC3
13	26.271	4.3	Hexanoic acid	142-62-1	$C_6H_{12}O_2$	116	FFNSC3
14	30.894	4.92	Octanoate <hexyl->	1117-55-1	$C_{14}H_{28}O_2$	228	FFNSC3

15	31.433	24.4	Butyrate <decyl->	1.09.5454	$C_{14}H_{28}O_2$	228	FFNSC3
16	33.861	2.01	Decanoic acid <n->	334-48-5	$C_{10}H_{20}O_2$	172	FFNSC3
17	34.294	10.38	Glucose pentaacetate	604-68-2	$C_{16}H_{22}O_{11}$	390	FFNSC3
18	51.743	7.52	Octacosane <n->	630-02-4	$C_{28}H_{58}$	394	FFNSC3
19	51.895	5.56	Pentacosane <n->	629-99-2	$C_{25}H_{52}$	352	FFNSC3
20	52.941	7.8	Phthalic acid, bis(2-ethylhexyl) ester (6CI,8CI)	117-81-7	$C_{24}H_{38}O_4$	390	FFNSC3
21	16.874	1.2	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	10230-62-3	$C_6H_8O_4$	144	NIST
22	10.062	8.76	Dimethylsulfoxonium formylmethylide	31043-74-0	$C_4H_8O_2S$	120	NIST
23	10.475	3.15	Methanesulfonyl chloride	124-63-0	CH_3ClO_2S	114	NIST
24	26.271	4.3	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	126-11-4	$C_4H_9NO_5$	151	NIST
25	30.894	4.92	3-Deoxyglucose	0-00-0	$C_6H_{12}O_5$	164	NIST
26	31.433	24.4	Decanoic acid, silver(1+) salt	13126-67-5	$C_{10}H_{19}AgO_2$	278	NIST
27	31.785	2.07	3-Deoxy-d-mannoic lactone	0-00-0	$C_6H_{10}O_5$	162	NIST
28	34.294	10.38	Beta.-l-Galactopyranosyl azide, 6-deoxy-2,3,4-triacetate	95581-07-0	$C_{12}H_{17}N_3O_7$	315	NIST
29	51.743	7.52	Bornane, 2-propionate-3-phenylcarbamate	0-00-0	$C_{20}H_{27}NO_4$	345	NIST
30	51.895	5.56	2,3-Di(2,2-dimethylethyl) thiophene-1,1-dioxide	128788-12-5	$C_{12}H_{20}O_2S$	228	NIST
31	52.941	7.8	1,3,5-Cycloheptatriene, 2,5-bis(tetrahydropyranyloxymethyl)-7,7-dimethyl-	0-00-0	$C_{21}H_{32}O_4$	348	NIST

In recent years, studies investigating the antiparasitic effects of plant extracts have been increasing rapidly. Studies on *Acanthamoeba* spp. are still ongoing. However, there is limited information on the mechanisms of action of phytocompounds responsible for the biological activity of plant extracts found to be effective in these studies.

Many researchers have used different parts of plants such as fruits, roots, flowers and leaves to produce ethanolic extract (Koohsari et al., 2015; Rodino et al., 2015; Ceylan et al., 2017; Yakoub et al., 2018; El-Beltagi et al., 2019; Kilic et al., 2019), antibacterial (Koohsari et al., 2015; Rodino et al., 2015; Ceylan et al., 2017; Yakoub et al., 2018; El-Beltagi et al., 2019; Kilic et al., 2019; Joma et al., 2020; Gurbuz et al., 2021) and antifungal (Rodino et al., 2015; Ceylan et al., 2017; El-Beltagi et al., 2019; Gurbuz et al., 2021) activities. There are also studies investigating antibacterial (Meric et al., 2014; Koohsari et al., 2015; Rodino et al., 2015; Gurbuz et al., 2021) and antifungal (Meric et al., 2014; Gurbuz et al., 2021) activities of *S. ebulus*.

In our study, the amoebicidal activity of the extract of *S. ebulus* was tested against *A. castellanii* trophozoites. It was found that *S. ebulus* extract showed a lethal effect on most of the trophozoites at the end of 24 and 48 hours at a concentration of 45 mg/mL, no viable trophozoites were found at 72 hours and a significant lethal effect at 72 hours at a concentration of 22.5 mg/mL (Figure 1). The IC_{50} values of the plant extract were 1.9, 3.3 and 4.8 mg/mL at 72, 48 and 24 hours, respectively (Table 1).

In addition to antibacterial and antifungal effects of the extracts prepared by some researchers using different parts of *S. ebulus* plant, there are also studies investigating the antiparasitic activity.

Gholami et al., (2013) investigated the scolisidal effect of methanolic fruit extract of *S. ebulus* on *Echinococcus granulosus* and emphasized that the extract showed a high scolisidal activity in vitro and therefore could be used in hydatid cyst surgery. Rahimi Esboei et al., (2013) investigated the anti-giardial activity of methanolic fruit extract of *S. ebulus* plant (at concentrations of 1, 10, 50 and 100 mg/mL) on *Giardia lamblia* cysts in vitro. They reported that *S. ebulus* extract at 100 mg/mL concentration had the highest anti-giardial activity compared to other concentrations.

Heidari-Kharaji et al., (2019) evaluated the immune responses in BALB/c mice infected with *Leishmania major* to determine the anti-leishmanial activity of the aqueous extract of *S. ebulus* and found that *S. ebulus* can significantly reduce the viability of *L. major* promastigotes and amastigotes.

In our study, the amoebicidal activity of the extract of *S. ebulus* against *A. castellanii* was tested. When the effects of the extract doses on the viability of the ethanolic leaf extract of *S. ebulus* on *A. castellanii* trophozoites were examined, it was shown that the viability rates decreased as the extract dose increased. The highest viability was measured at the lowest dose of 1.4 mg/mL and the lowest viability was measured at the highest dose of 45 mg/mL. When the effects of application times on viability were examined, it was determined that the viability rates decreased as the waiting time increased. The highest viability was measured at the end of 24th hour, while the lowest viability was measured at the end of 72nd hour (Table 2).

In the literature study, they published that the ethanolic leaf extract of *Morinda citrifolia* has phytochemicals such as octanoic acid, sorbitol, cyclopropyl, hexanoic acid, n-decanoic acid, allantoin, mannitol, glycerin, and gamma-tocopherol (Rivera et al., 2012) which are chemical compounds of medicinal importance. El-Beltagi et al., (2019) investigated the chemical compounds, antioxidant and anti-microbial activities of extracts from leaves and fruits of *Ficus sycomorus* using ethanolic and ethyl acetate. Shibula and Velavan, (2015) emphasized that methanolic extract of *Annona muricata* leaves and ethanolic extracts of leaves and fruits of *Trichosanthes dioica* Roxb Kavitha, (2021) are candidate plants whose phytocompounds may have antioxidant and anti-microbial, anti-cancer, anti-diabetic activities. In our study, chemical constituents of *S. ebulus* were determined by GC-MS analysis. Dimethylsulfoxonium formylmethylide was found in the essential oil obtained from *Jasminum sambac* Jha et al., (2022) 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one was also found in *Annona muricata* leaves (Shibula and Velavan, 2015).

There are studies investigating the cytotoxicity Meric et al., (2014) and DNA protective activities George et al., (2015), Ozaslan et al., (2016), Kilic et al., (2019), Joma et al., (2020) of extracts obtained from plants in mammalian cells. In this study, the cytotoxicity and DNA protective activity of *S. ebulus* extract in mammalian cells were determined. The cytotoxicity of ethanolic leaf

extract of *S. ebulus* in mammalian cells was examined using the HeLa cell line. As a result, while the leaf extract of *S. ebulus* showed amoebicidal activity on *A. castellanii* trophozoites, it was determined that its cytotoxicity in mammalian cells decreased dose-dependently towards lower concentrations (Figure 4).

When the DNA protective activity of the ethanolic leaf extract of *S. ebulus* (Figure 5) was examined, it shown that high concentrations (45, 22.5 and 11.25 mg/mL) had weak effects on preventing DNA damage caused by (-OH), but low concentrations (5.62, 2.8 and 1.4 mg/mL) had a strong effects on preventing DNA damage caused by (-OH).

4. Conclusions and Recommendations

With the data obtained, the amoebicidal activity of the ethanolic leaf extract of *S. ebulus* against *A. castellanii* trophozoites was found. It was shown that as the extract's dose increased, the parasite's survival rate decreased. When the effects of application times on viability were examined, it was shown that the survival rates of the parasite decreased as the waiting time increased. Additionally, it is observed that *S. ebulus* extract reduces cell viability in the HeLa cell line depending on the dose. On the other hand, it has been determined that high doses of *S. ebulus* extract have weak effects on preventing DNA damage, but low doses of the extract have strong effects on preventing DNA damage.

The study showed that different concentrations of the extract significantly reduced the survival rate of *A. castellanii* and the effect increased as the dose increased. Additionally, the extract was found to have the potential to protect against DNA damage at low concentrations and exhibited dose-dependent cytotoxic effects in the HeLa cell line. These findings suggest that *S. ebulus* leaf extract has the potential to be used as an alternative treatment for infections caused by *Acanthamoeba*. The data obtained show that the extract offers an innovative treatment approach by focusing on natural components that are both effective against parasites and exhibit DNA-protective and noncytotoxic properties, and can be investigated in clinical applications and can be a potentially safe alternative or complementary treatment option.

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Authors' Contributions

The authors declare that they have contributed equally to the article.

Statement of Conflicts of Interest

The authors of the article declare that there is no conflict of interest between them.

Statement of Research and Publication Ethics

The authors declare that this study complies with Research and Publication Ethics.

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