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Investigation of Antiproliferative Effects of *Heracleum persicum* Extracts on Glioblastoma Multiforme Cell Line

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ABSTRACT

Heracleum persicum (*H. persicum*) is a plant known for its anti-inflammatory, analgesic, anticonvulsant, and antioxidant properties. In this study, the antiproliferative effects of ethanolic leaf and root extracts of the plant on human Glioblastoma Multiforme U-87MG cell line were evaluated. The leaf and root extracts were applied at concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mg/mL for 24 and 72 hours, and cell viability was determined using MTT assay. The results showed that at a concentration of 10^{-1} mg/mL for 24 hours, the leaf and root extracts caused cell death at rates of 76% and 81%, respectively. In the 72-hour application, the leaf extract at a concentration of 10^{-1} mg/mL led to 83% cell death, while the root extract at the same concentration caused 92% cell death. These findings demonstrate that *H. persicum* extracts exhibit antiproliferative effects on U-87MG cell line and suggest their potential as therapeutic agents.

Keywords: Antiproliferative effect, Cancer cells, Glioblastoma multiforme, *Heracleum persicum*, Plant extracts

Heracleum persicum Ekstraktlarının Glioblastoma Multiforme Hücre Hattı Üzerindeki Antiproliferatif Etkilerinin Araştırılması

ÖZ

Heracleum persicum (*H. persicum*), anti-inflamatuvar, analjezik, antikonvülzan ve antioksidan özellikleriyle bilinen bir bitkidir. Bu çalışmada, bitkinin etanolik yaprak ve kök ekstrelerinin insan Glioblastoma Multiforme U-87MG hücre hattı üzerindeki antiproliferatif etkileri değerlendirildi. Yaprak ve kök ekstreleri 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} ve 10^{-6} mg/mL konsantrasyonlarında 24 ve 72 saat süreyle uygulanarak hücre canlılıkları MTT testi ile belirlendi. Sonuçlar, 10^{-1} mg/mL konsantrasyonunda 24 saatte yaprak ve kök ekstrelerinin sırasıyla %76 ve %81 oranında hücre ölümüne neden olduğunu gösterdi. 72 saatlik uygulamada, yaprak ekstresinin 10^{-1} mg/mL konsantrasyonu %83 oranında hücre ölümüne yol açarken, kök ekstresinin 10^{-1} mg/mL konsantrasyonu %92 oranında hücre ölümüne neden oldu. Bu bulgular, *Heracleum persicum* ekstrelerinin U-87MG hücre hattında antiproliferatif etki gösterdiğini ve potansiyel terapötik ajanlar olarak değerlendirilebileceğini ortaya koymaktadır.

Anahtar Kelimeler: Antiproliferatif etki, Bitki ekstraktları, Glioblastoma multiforme, *Heracleum persicum*, Kanser hücreleri

INTRODUCTION

Cancer is a disease of multifactorial etiology that entails abnormal cell growth and metastasis. Some of the causative factors are genetic mutations, environmental and lifestyle. If not detected early, it may spread to other organs and make the process of treatment complex. Cancer is one of the most severe threats to public health at present, affecting the lives of millions of people. Therefore, understanding the biological mechanisms of cancer, developing new forms of treatment and implementing preventive methods are of utmost importance [1]. According to data from the United States Central Brain Tumor Registry between 2013 and 2017, Glioblastoma Multiforme (GBM) comprises 45% of all primary brain and other central nervous system tumors

and accounts for 48.6% of malignant tumors. GBM is more frequently observed in white populations and males compared to females, with its incidence increasing with age Since 1940, GBM has been classified by Hans-Joachim into two subtypes: primary and secondary. Primary GBM constitutes 90-95% of all GBM cases, characterized by rapid development of clinical symptoms within the first 3 months. Secondary GBM represents 5-10% of cases, typically occurring in younger individuals, with symptoms appearing over several months to a year [2]. Initial symptoms observed in GBM-diagnosed patients include increased intracranial pressure, headache, neurological disorders, visual disturbances, speech impairments, nausea, vomiting, memory loss, and seizures. These symptoms result from elevated pressure due to rapid tumor growth, with clinical manifestations varying according to the tumor's anatomical location [3]. Each year, approximately 5-6 cases per 100,000 individuals are diagnosed with primary malignant brain tumors, about 80% of which are malignant gliomas (MG). GBM accounts for the majority of MG cases and exhibits high mortality and morbidity rates [4]. Surgical resection is the primary treatment method. When surgery is not feasible, radiotherapy or chemotherapy is employed. After surgical resection, despite multimodal treatments including radiotherapy and chemotherapy, median survival is 12-15 months and the 5-year survival rate is approximately 7.2% [5]. Chemotherapy and pharmacotherapy are administered to GBM cells distributed in various brain regions post-surgery. Temozolomide, Carmustine, Lomustine, Bevacizumab, and Regorafenib are currently used widely, either individually or in combination, for GBM treatment [6]. Despite these therapeutic advances, the prognosis for GBM patients remains dismal, highlighting the necessity for continued research into novel therapeutic agents with improved efficacy, specificity, and safety profiles [7]. Plant-based compounds, in particular, have garnered increasing attention for their pharmacological versatility and lower toxicity compared to synthetic chemotherapeutics [8]. Natural products have historically served as the basis for many cancer therapies, and emerging studies support their potential in targeting key oncogenic pathways in GBM [7]. GBM has a poor prognosis due to limited blood-brain barrier penetration, significant side effects, and inadequate specificity to brain cancer cells. Consequently, there is an ongoing search for alternative therapeutic approaches [9]. One study reported that nano-drug delivery systems loaded with Metformin and Irinotecan exhibited greater anticancer efficacy against GBM compared to standard therapies without carriers [10]. Another study demonstrated molecular treatment strategies for GBM using the CRISPR/CAS9 gene-editing technique and T-cell-based immunotherapies [11]. In a botanical study conducted in 2019, Curcumin was shown to target multiple signaling pathways involved in glioblastoma invasiveness and drug resistance, including those associated with glioma stem cell activity [12]. A review published in 2020 indicated that Quercetin exhibited antitumor and antiproliferative activities on GBM cells, potentially playing a significant role in GBM treatment [13]. In another botanical study, the essential oil and hydroalcoholic extract of *H. persicum* demonstrated significant anti-inflammatory and analgesic effects by notably reducing abdominal contractions and pain responses induced by acetic acid [14]. Furthermore, in an experimental seizure model, dose-dependent anticonvulsant activity was observed with *H. persicum* seeds [15]. In the present study, the antiproliferative effects of *H. persicum* leaf and root extracts on the human GBM U-87MG cell line were investigated. This study is one of the few that comparatively evaluates both the leaf and root components of *H. persicum* on a GBM cell line, thus contributing to the limited oncobotanical research in this area. The major objective of this study was to evaluate the antiproliferative effects of ethanoic leaf and root extracts of the plant on GBM cell line. For this purpose, the effects of utilizing extracts at different concentrations on cell viability were investigated. The sub-objectives of the study were to compare leaf and root extracts for their cytotoxic potentials, to determine how the time of application (24 and 72 hours) influenced the viability of the cells and to investigate the dose-dependent effects through MTT test. The results may serve as a foundational step toward future mechanistic studies, including apoptosis assays, molecular pathway profiling, and *in vivo* validations. Data obtained assisted in evaluating the plant for its potential as a biological agent in cancer management.

MATERIAL and METHODS

2.1. Collection and Drying of *H. persicum* Plant

The *H. persicum* plant was collected in Van in May 2022. It was dried in a clean environment at room temperature, away from direct sunlight. The collection and drying of *H. persicum* were carried out by our research team.

2.2. Preparation of *H. persicum* Plant Extract

Obtaining extracts from the leaves and roots of *H. persicum* was carried out by making minor modifications based on the method proposed in the study of Zheleva-Dimitrova, et al. [16]. Briefly, the root and fruit of the plant, dried in the shade, were ground into powder using a grinder. A total of 150 g of powdered *H. persicum* root and fruit was macerated overnight in 1.5 L methanol. After maceration, extraction was carried out at room temperature for 24 hours using a mixer. This extraction procedure was repeated three times. After each extraction, the extracts were filtered, and the filtrates combined and evaporated to dryness using a rotary evaporator at 40 °C and 120 rpm. Finally, the extracts were dissolved in water and lyophilized.

2.3. Cell Culture

2.3.1 Preparation of Cell Culture Medium

89 mL ready-made cell culture medium (RPMI-1640), 10 mL Fetal Bovine Serum (FBS), 1 mL Penicillin+Streptomycin+Amphotericin B substances

were added to obtain a ready-made cell culture medium. That ratio was used for whole experiment [17].

2.3.2 Cell Incubation Conditions

Cells obtained from ATCC HTB-14™ were used. These cells were incubated in ready-made medium (RPMI-1640) in a 5% CO_2 and 37°C oven (Nuaire) in 25 cm^2 flasks under sterile conditions. Thus, the cells were allowed to proliferate [18].

2.3.3 Subculturing of Cells

When the reproducing cell passages reached 80-90% confluent, they were re-passaged. For this purpose, when the reproducing cells reached the desired majority; the medium in the flasks was taken with a pipette and washed with sterile phosphate buffer solution (PBS) (2 mL for 25 cm^2). Then, the PBS was removed with a pipette and 3 mL of trypsin-EDTA solution was added to remove the cells from the area where they adhered and kept in the oven for 10 minutes. After removing the cells that adhered to the entire surface, the suspended cell + trypsin-EDTA solution was taken into a 15 mL tube. Medium twice the volume of trypsin was added to the solution for trypsin inhibition. The suspension in the tube was centrifuged at 1300 rpm for 10 minutes, then the trypsin-EDTA solution was removed, and the cells were suspended with fresh cell medium and divided into 3 25 cm^2 flasks; it was re-passaged. The re-passaged cells were incubated at 37°C in a 5% CO_2 environment.

2.3.4 Freezing and Storage of Cells

The cells lifted from the surface with trypsin-EDTA were centrifuged and the trypsin was removed from the medium. Then the cell pellet was diluted with 1 mL medium and the cell count was performed. Trypsin was inhibited with at least twice the volume of serum medium. The cells were pipetted into a single cell suspension and transferred to a falcon tube. 2-3 mL more medium will be added. The cell suspension was centrifuged at 1300 rpm for 10 minutes and the supernatant was removed. The pellet was diluted with 900 µL of cell medium and counted. The cell solution suspended with 100 µL of dimethyl sulfoxide (DMSO) and 900 µL of cell medium was added to the freezing tubes. The tubes were placed in the freezing container and stored in the deep freezer at -80°C to be used when needed.

2.3.5 Calculation of Cell Numbers

10 µL of the cell suspension diluted in 1 mL of culture medium was taken and placed in an Eppendorf tube and 90 µL of trypan blue dye was added and mixed. This mixture was placed on a Neubauer slide and the cells in 5 compartments were counted. This number found was multiplied by the dilution amount of 50,000. As a result, it was found how many million cells there were in 1 mL of medium. Thus, the number to be seeded was determined and the cells were seeded in petri dishes. $5x10^6$ cells were transferred to a 100 mm culture petri dish and 5000 cells were transferred to each well of a 96-well culture plate.

2.4. Cell Proliferation Analysis

2.4.1. MTT Method

MTT assay is a method in which the amount of cell growth is determined by the colorimetric determination of formazan dyes or enzymatic activity due to MTT reduction. With this method, the cytotoxic or proliferative effects of any therapeutic agent to be used on the cell can be determined. The method is based on the principle of colorimetric determination of the color change in cells incubated with MTT agent. The resulting color change is the result of the decrease in tetrazolium salt in the active cell mitochondria of formazan salts colored yellow. The absorbance value of these compounds is proportional to the determination of their metabolic activities [19, 20].

2.4.2. Method of Application

1 day before the application of the MTT method, 5000 cells (5000/well) were counted in a 96-well plate and 100 µL medium was prepared and planted in the wells. The microplate was kept in an incubator set at 37°C and 5% CO_2 for 24 hours, and the cells were allowed to adhere to the surface. In this way, the damage caused by the trypsin enzyme on cell membrane proteins and 27 growth factor receptors was eliminated. A 24-hour period is required for the re-synthesis of these proteins and growth factors. In addition, in this way, the cells are provided with metabolic activity before the treatment of therapeutic agents in the medium with the cells. After 24 hours of incubation, the plant extracts prepared in serial dilutions was added to the cell line medium divided into groups in series. (100 µL of diluted extract was added to the 100 µL medium and cell mixture and its concentration was adjusted). The first column of the A series was set as cell control and the second column as medium control. The MTT method was applied to the cell line to which the extract was applied after 24 and 72 hours of incubation [21].

2.5 Statistical Analysis

All experiments were performed in triplicate and the experimental results on cytotoxic activities of *H. persicum* leaf and root extracts show the means ± standard deviation (SD). Statistical analyses were performed using the One-Way ANOVA Duncan test in the IBM SPSS Statistics 20 program. p <0.05 was considered significant at the significance level. The graphs and IC_{50} values were created, edited and determined using GraphPad Prism 8.0 software.

RESULT and DISCUSSION

Plant extracts could emerge as promising candidates in GBM research due to their diverse bioactive compounds, which exhibit antiproliferative, pro-apoptotic, and anti-inflammatory properties. Many phytochemicals, such as flavonoids, alkaloids, and polyphenols, have

demonstrated the ability to modulate key signaling pathways involved in GBM progression, including those regulating cell cycle arrest, oxidative stress, and angiogenesis. Moreover, plant-derived compounds often show lower toxicity compared to conventional chemotherapeutics, making them attractive for combination therapies to enhance treatment efficacy while minimizing side effects. The main aim of this study was to evaluate the effects of the plant extracts on GBM cell line after certain time intervals. Here, it was planned to evaluate the variation that occurred with time by treating the cells with the extracts for different time intervals. It was hence tried to determine whether the antiproliferative effect of the extracts was time dependent and at which time they were most effective. In the 24-hour treatment, the 10^{-1} mg/mL dose resulted in 76% cell death compared to the control and other groups. The IC_{50} value for the 24-hour period was found to be 33 µg/mL. Other groups did not cause statistically significant cell death compared to the control group (Fig 1).

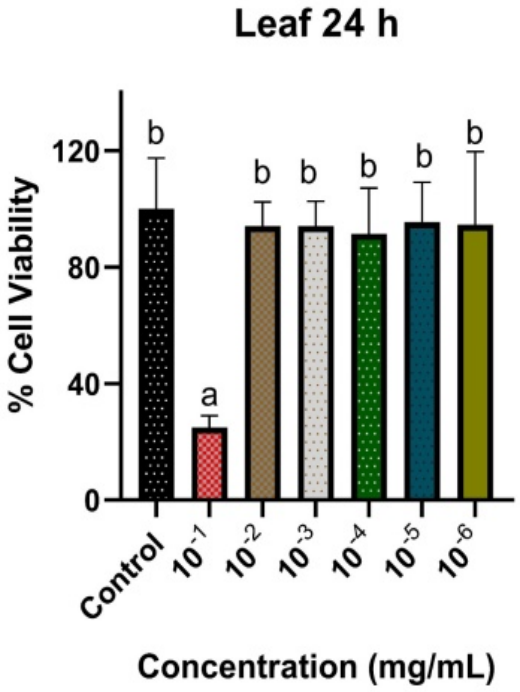

Figure 1. Cell viability (% control) in U-87 MG human glioblastoma cells after 24 h incubation with different concentrations of *H. Persicum* leaf extract. a, b: Groups with different letters indicate statistically significant differences when compared to each other.

In the 72-hour treatment, similarly, the 10^{-1} mg/mL dose led to statistically significant cell death of 83% compared to the control group. The IC_{50} value at 72 hours was also found to be 46 µg/mL. Other doses demonstrated effects similar to the 24-hour treatment, and additionally, cell viability-enhancing effects were observed at lower doses (Fig 2).

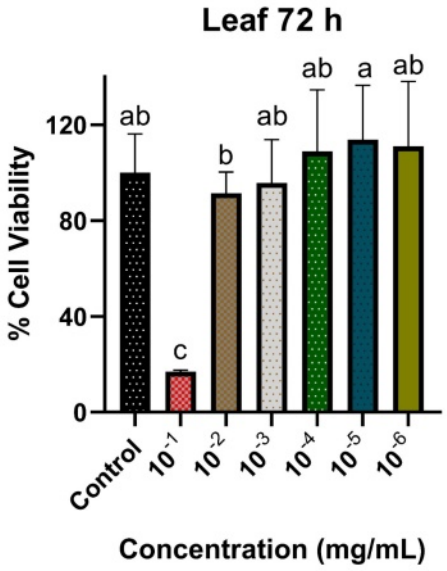

Figure 2. Cell viability (% control) in U-87 MG human glioblastoma cells after 72 h incubation with different concentrations of *H. Persicum* leaf extract. a, b, c: Groups with different letters indicate statistically significant differences when compared to each other.

In the 24-hour treatment, the 10^{-1} mg/mL dose was statistically significant compared to the control and other groups, resulting in 81% cell death. The IC_{50} value for 24 hours was 86 µg/mL. Other doses were not statistically significant (Fig 3).

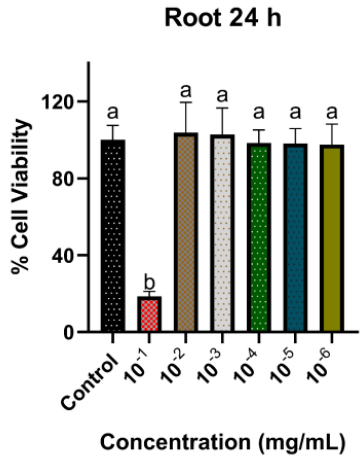

Figure 3. Cell viability (% control) in U-87 MG human glioblastoma cells after 24 h incubation with different concentrations of *H. Persicum* root extract. a, b: Groups labeled with different letters indicate statistically significant differences compared to each other.

In the 72-hour treatment, similarly, the 10^{-1} mg/mL dose led to statistically significant cell death of 92% compared to the control group. The IC_{50} value for 72 hours was determined as 11 µg/mL (Fig 4). Groups labelled with different letters indicate statistically significant differences compared to each other.

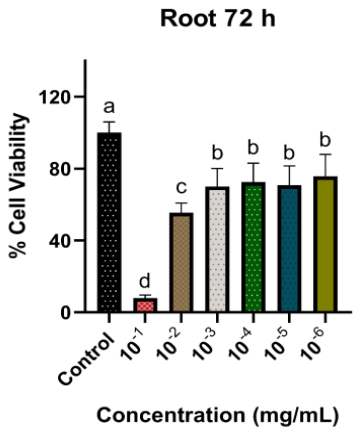

Figure 4. Cell viability (% control) in U-87 MG human glioblastoma cells after 72 h incubation with different concentrations of *H. Persicum* root extract. a, b, c, d: Groups labeled with different letters indicate statistically significant differences compared to each other.

Each year, approximately 440,000 individuals worldwide are diagnosed with brain tumors, and 80% of these cases are GBM. GBM is classified as the most severe form of primary brain tumors by the Alifieris & Trafalis, 2015. The World Health Organization classifies GBM based on glial cell origin, proliferation rate, vascularization, presence of necrosis, and response to treatment [22]. Previous studies have reported that *H. persicum* possesses various pharmacological activities, including anti-inflammatory, analgesic, anticonvulsant, and antioxidant effects [23]. Sayyah et al. reported anticonvulsant activity in a dose-dependent manner in *H. persicum* seeds using experimental seizure models. Hajhashemi et al. reported that the essential oil and hydroalcoholic extracts of *H. persicum* significantly reduced pain response and demonstrated anti-inflammatory and analgesic activities. However, the anticancer effects of *H. persicum* on GBM cells have not been previously investigated. This study evaluated the anticancer effects of leaf and root ethanolic extracts of *H. persicum* against the human GBM U-87MG cell line. The MTT assay was employed to assess cell viability. This method detects viable cells by reducing $NADPH^+$ to $NADP^-$, forming formazan crystals from the tetrazolium salt [24]. The crystals are dissolved in dimethyl sulfoxide (DMSO), producing a blue-purple color measurable at an optical density of 570 nm [25]. In this study, the most effective doses of *H. persicum* root and leaf extracts ranged between 10 µg/mL and 100 µg/mL. The IC_{50} values of the leaf extracts at 24 and 72 hours were 33 µg/mL and 46 µg/mL, respectively, whereas the IC_{50} values of the root extracts at 24 and 72 hours were 86 µg/mL and 46 µg/mL, respectively. Therefore, the most promising antineoplastic activity was observed with the root extract after 72 hours of exposure. Further fractionation and purification of the root extract through various methods may reveal potential active pharmaceutical compounds. Future studies investigating potential synergistic effects of these compounds, their pharmacokinetic profiles, and their underlying antineoplastic mechanisms would enhance understanding of the therapeutic potential of this extract.

CONCLUSION

In conclusion, leaf and root extracts of *H. persicum* demonstrated antiproliferative effects on the U-87MG cancer cell line, potentially via apoptotic or oxidative stress pathways. To the best of our knowledge, there are no prior studies specifically evaluating the antiproliferative effects of *H. persicum* root and leaf extracts on glioblastoma cell viability. To better understand the molecular mechanisms underlying this process, further in vivo and in vitro studies are crucial. These studies can enlighten us on the intricate biochemical processes involved, collecting relevant information on their functional importance. Through conducting extensive research at cellular and organismal levels, scientists can refine therapeutic approaches, enhance drug development techniques, and determine potential targets for medical treatment.

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