

***Hedera helix* (Wall Ivy) leaf ethanol extract shows cytotoxic and apoptotic effects in glioblastoma cells by generating reactive oxygen species^{*}**

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ABSTRACT

Aim: Glioblastoma, known for its aggressiveness, accounts for most malignant gliomas and the efficiency of its treatment is still not enough showing remarkably poor prognosis even though a complex treatment approach. *Hedera helix* is an evergreen plant mostly known for its effects on respiratory function, especially in chronic bronchial asthma, as well as its antimicrobial, anti-inflammatory, antioxidant, and potential antitumor properties, while its effect on glioblastoma cancer and the underlying mechanism has not been elucidated yet. The aim of this study was to examine the anti-cancer properties of *Hedera helix* (common ivy) in relation to its ability to induce cytotoxicity, apoptosis, and reactive oxygen species production in glioblastoma (U87) cells.

Material and Methods: The leaves' ethanol, methanol, and water extracts were analyzed for phenol, flavonoid, and antioxidant levels through photometric methods. Then, different concentrations of ethanol extract were applied to U87 cells for 24 hours and analyzed for cytotoxic, apoptotic, and ROS-generating effects by measuring the luminometric intracellular ATP amount, Acridine Orange/Ethidium Bromide double staining, and DCFH-DA methods, respectively.

Results: Phenol, flavonoid, and antioxidant results of all three extracts show that ethanol was best for extraction. The results showed that *Hedera helix* ethanol extract had dose-dependent cytotoxic and apoptotic effects with increased intracellular ROS levels.

Conclusion: These results concluded that high doses of *Hedera helix* ethanolic extract may exhibit anti-cancer effects through pro-oxidant activity.

Keywords: *Hedera helix*, common ivy, anti-cancer, pro-oxidant, glioblastoma.

INTRODUCTION

Glioblastoma is the prevailing and highly aggressive form of primary brain tumor in adults, comprising 57.3% of all gliomas [1] and 45.6% of all primary malignant brain tumors [2]. Glioma is the most prevalent malignant neoplasm of the central nervous system (CNS) when considering incidence and mortality rates [3]. The incidence of glioblastoma multiforme (GBM) tends to increase with age [4], and the occurrence is increasing potentially due to multifactorial factors, including an aging population, exposure to ionizing radiation, air pollution, and other possible causes [5]. Although surgical resection, radiotherapy (RT), and simultaneous chemotherapy are applied in conventional treatment [6], the success rate is low, the side effects are high and the survival time is around 15 months. [7]. Therefore, the search for alternative treatments continues, and one of them is the use of medicinal plants, described as phytotherapy.

Hedera helix L. (*H. helix*), commonly known as English ivy or common ivy, is an evergreen woody vine belonging to the family Araliaceae, and one of 15 species in the *Hedera* genus [8]. *H. helix* has a rich historical background in traditional medicine, where it has been widely utilized to address a diverse range of health conditions including respiratory infections, arthritis, and skin disorders, among others. [8]. The *Hedera* leaves have been used for treating cough and respiratory problems and the German Commission E has approved the use of *H. helix* for the treatment of catarrhs (excessive mucus production) affecting the respiratory tract and for alleviating symptoms associated with chronic inflammatory bronchial conditions [9]. *H. helix* extract is also often used in cosmetics and skin care products due to its emollient and antipruritic properties. Creams, lotions, and shampoos containing *H. helix* extract are commonly used to soothe and moisturize dry, pruritic skin and to relieve symptoms of skin disorders such as eczema and psoriasis [10]. In homeopathy, the *H. helix* is believed to have therapeutic properties that can be used to treat a range of conditions, including hyperthyroidism, rheumatic disorders, and respiratory tract inflammation [8]. Anti-inflammatory, antimicrobial, and anthelmintic activities of the *H. helix* were shown in various studies in the literature [11-13]. Besides all these

pharmacological effects, the anti-tumor effects of *H. helix* extracts also have been investigated and shown [14-16]. However, previous studies revealed a lack of research on the potential anti-tumor effects of *H. helix* on glioblastoma. Therefore, the current investigation was carried out to fill this knowledge gap and explore the potential cytotoxic, apoptotic, and Reactive Oxygen Species (ROS) production effects of *H. helix* in the U87 glioblastoma cell line.

MATERIAL AND METHODS

Cell culture

U87 cells were sourced from the American Type Cell Culture Collection (ATCC, Germany). These cells were regularly maintained in DMEM culture medium (Biochrom, Germany) supplemented with 10% fetal bovine serum (Biochrom, Germany) and 1% penicillin/streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) (Biochrom, Germany). The cells were cultured in a humidified atmosphere with 5% CO₂ at a temperature of 37°C.

Plant extraction

The ivy leaves collected from the inner Anatolia region were subjected to extraction using ethanol, methanol, and water. The dried samples (100 g) were mechanically powdered and then extracted either with 70% ethanol (Absolute; Merck, Germany), 70% methanol (Merck, Germany), or 100% water. The extraction processes were carried out on an orbital shaker at room temperature for a minimum of 24 hours. The resulting ethanol, methanol, and water extracts were filtered using Whatman filter paper, and the alcohol was removed using a rotary evaporator (Heidolph, Germany). The remaining supernatant was subsequently lyophilized under a vacuum at -82°C using a freeze dryer (Labconco, USA).

Total phenolic and flavonoid contents

The phenolic and flavonoid contents of the ethanol, methanol, and water extracts were assessed using photometric methods. The total phenolic contents (TPC) were measured using the Folin-Ciocalteu method with slight modifications [17]. To determine the TPC, the absorbance of the samples

was measured at a wavelength of 760 nm using a spectrophotometer (Varioskan Flash, Multimode Reader, Thermo Scientific, USA). A standard curve was created by using various concentrations of gallic acid. The TPC values were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry plant material (mg GAE/g plant material).

To quantify the total flavonoid compounds (TFC), a colorimetric assay was performed according to the described method [18, 19]. After the addition of 75 μ L of 5% sodium nitrite (NaNO_2) to the mixture of 250 μ L sample and 1 mL of distilled water in a volumetric flask, 75 μ L of 10% aluminum chloride (AlCl_3) was added after 5 minutes. Subsequently, after 6 minutes, 500 μ L of 1 N sodium hydroxide (NaOH) was added to the mixture. Following the addition of 500 μ L of 1 N sodium hydroxide (NaOH) to the mixture, it was diluted with 2.5 mL of distilled water and the resulting samples were vortexed and kept at room temperature. The absorbance of the resulting mixture was measured directly at 510 nm using a Varioskan Flash Multimode Reader. A calibration curve was established using a diluted solution of quercetin to determine the TFC. The TFC values were expressed in milligrams of quercetin equivalent per 100 grams of dry matter (mg QuE/100 g DM).

DPPH assay

The DPPH assay is commonly used to measure the ability of a substance to scavenge free radicals. This is done by observing the reduction in the absorption of a methanolic solution of DPPH, a stable radical after it is neutralized by an antioxidant. This is indicative of the antioxidant's radical scavenging activity. To carry out the assay, 20 μ L of diluted extract is added to 980 μ L of 100 μ M DPPH solution, followed by a 30-minute incubation period. The absorbance of the resulting mixture is then measured at 517 nm using a microplate reader (Varioskan Flash, Multimode Reader, Thermo Scientific, America). The percentage inhibition of the DPPH radical was determined using the following formula: $I = 100 (A_c - A_s)/A_c$, where I represents the percentage of DPPH inhibition, A_c is the absorbance value of the control sample, and A_s is the absorbance value of the sample being tested [20, 21].

ATP Cell Viability Assay

Cytotoxicity of *H. helix* ethanol extract on glioblastoma cells investigated through ATP cell

viability assay. In this assay, ATP is measured using a luciferase-based assay system. The assay relies on the fact that luciferase, an enzyme found in fireflies, catalyzes the reaction between ATP and luciferin, resulting in the emission of light. The amount of light emitted is proportional to the amount of ATP present in the sample. For performing the ATP test, 5×10^3 U87 cells were plated in an opaque white 96 well plate and treated with different concentrations of ethanol extract for 24 hrs. Then, the ATP cell viability kit (Promega, USA) was applied according to the manufacturer's protocol. Luminometric light intensity was measured through a multiscan plate reader (Varioskan Flash, Multimode Reader, Thermo Scientific, USA), and intracellular ATP levels were determined. Cell viability of extract-treated cells was compared to non-treated cells and half maximal inhibitory concentration (IC_{50}) of the extract was calculated.

DCFH-DA for ROS analysis

DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate, is a fluorescent dye that is frequently utilized to quantify the levels of intracellular ROS. When DCFH-DA is taken up by cells, it is hydrolyzed by intracellular esterase to form the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH). DCFH is oxidized by ROS and forms the 2',7'-dichlorofluorescein (DCF) which is a fluorescent compound. The intensity of the DCF fluorescence is proportional to the amount of ROS generated in the cell. To perform ROS analysis using DCFH-DA, 5×10^3 U87 cells were plated in a black 96 well plate and treated with the same concentrations of the ethanol extract used in the cytotoxicity test for 24 hrs. Then the cells were washed with 1xPBS one time and incubated with 5 μ M H₂DCF-DA for 30 minutes at 37°C in the dark. After 30 min. incubation, the absorbance was measured using a multi-plate reader (Varioskan Flash Multimode Reader, Thermo Scientific) to measure the DCF fluorescence intensity. At excitation and emission wavelengths 485 nm and 535 nm, respectively. The results were normalized to the viable cells.

Acridine Orange/Ethidium Bromide (AO/EB), Double Staining Method

The apoptotic effect was studied by AO/EB technique which is a simple and inexpensive method used to differentiate apoptotic cells from living cells. According to the cytotoxicity

experiments, the concentrations around and below the IC_{50} value (300, 350, 400, 450, and 500 $\mu\text{g/ml}$) were applied to the cells for 24 hours. The cells were then removed from the plates and stained with the AO/EB dye (Sigma Aldrich, USA). Stained cells were examined through the fluorescent microscope (Leica Microsystems, Germany) and apoptotic cell ratios were determined by evaluating at least 100 cells for each sample. Green-dyed cells were counted as live cells due to AO staining and dead cells were red due to EB staining.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean value \pm standard deviation (mean \pm SD). The conformity of the quantitative variables to the normal distribution was examined using the Shapiro Wilk test. One-way analysis of variance (One-Way ANOVA) was used to compare the mean of related variables between groups. The Dunnett test was used as a post hoc analysis for pairwise comparisons. The statistical significance level was taken as 0.05 and the calculations were made using IBM SPSS Statistics for Windows, Version 26.0. (Armonk, NY IBM Corp).

RESULTS

Comparison of ethanol, methanol, and water extracts for antioxidant activity with phenolic and flavonoid contents

The extracts were prepared using three different solvents; ethanol, methanol, and water, and their TPC, TFC, and antioxidant capacity were compared to choose the best extraction solvent. Our results showed us that phenolic and flavonoid content was highest in ethanol extraction of the ivy (Figure 1).

The antioxidant activity of the extracts was analyzed by DPPH assay which is commonly used to assess the ability of antioxidants to scavenge free radicals. The disappearance of the DPPH absorption reflects the antioxidant's ability to react with the radical. Therefore, a higher reduction in the DPPH absorption indicates a stronger antioxidant effect. Our DPPH assay results showed that higher antioxidant activity belonged to the ethanol extract of the ivy that was also compatible with the extracts' phenolic and flavonoid contents (Figure 1).

Cytotoxicity of *H. helix* extract on glioblastoma cells and apoptosis

Cytotoxicity analysis of *H. helix* ethanol extract was carried out using the ATP cell viability method. ATP assay, based on the measurement of ATP levels in cells, is a good indicator of cell viability and a widely used method for evaluating cytotoxicity. The viability test demonstrated that the *H. helix* extract had a dose-dependent cytotoxic effect, and the IC_{50} was approximately 475 $\mu\text{g/ml}$ (Figure 2).

Apoptosis-inducing effect of the extract was studied using the AO/EB double staining method. This method is a frequently used technique to analyze apoptosis since it is fast, cheap, and easy to perform. The AO/EB assay results indicated that the ethanol extract of *H. helix* leaves induced apoptosis in glioblastoma cells at doses approximately equal to or below the IC_{50} . Furthermore, the level of apoptosis increased with higher concentrations of the extract (Figure 3).

ROS Generating effect of *H. helix*

ROS are molecules that are naturally produced within cells during metabolic processes. While ROS plays important roles in various cellular functions, including signaling and regulation of gene expression, high levels of ROS can cause cellular damage, leading to cell death. In the current study, we also investigated the connection between *H. helix* and ROS generation. DCFH-DA results showed that high doses of *H. helix* increased intracellular ROS levels in glioblastoma cells and exhibited pro-oxidant activity even though the plant has antioxidant properties with lower doses (Figure 4).

DISCUSSION

Glioblastoma is a highly aggressive and malignant type of brain tumor that originates from the astrocytes, which are supportive cells in the brain. It is the most common and deadliest form of primary brain tumor [22]. Due to the aggressive nature of glioblastoma, there is ongoing research aimed at developing new treatments for the disease, including immunotherapy, targeted therapies, and gene therapy [23, 24].

Over the past few decades, there has been an exponential increase in the field of herbal medicine, and it is becoming increasingly popular

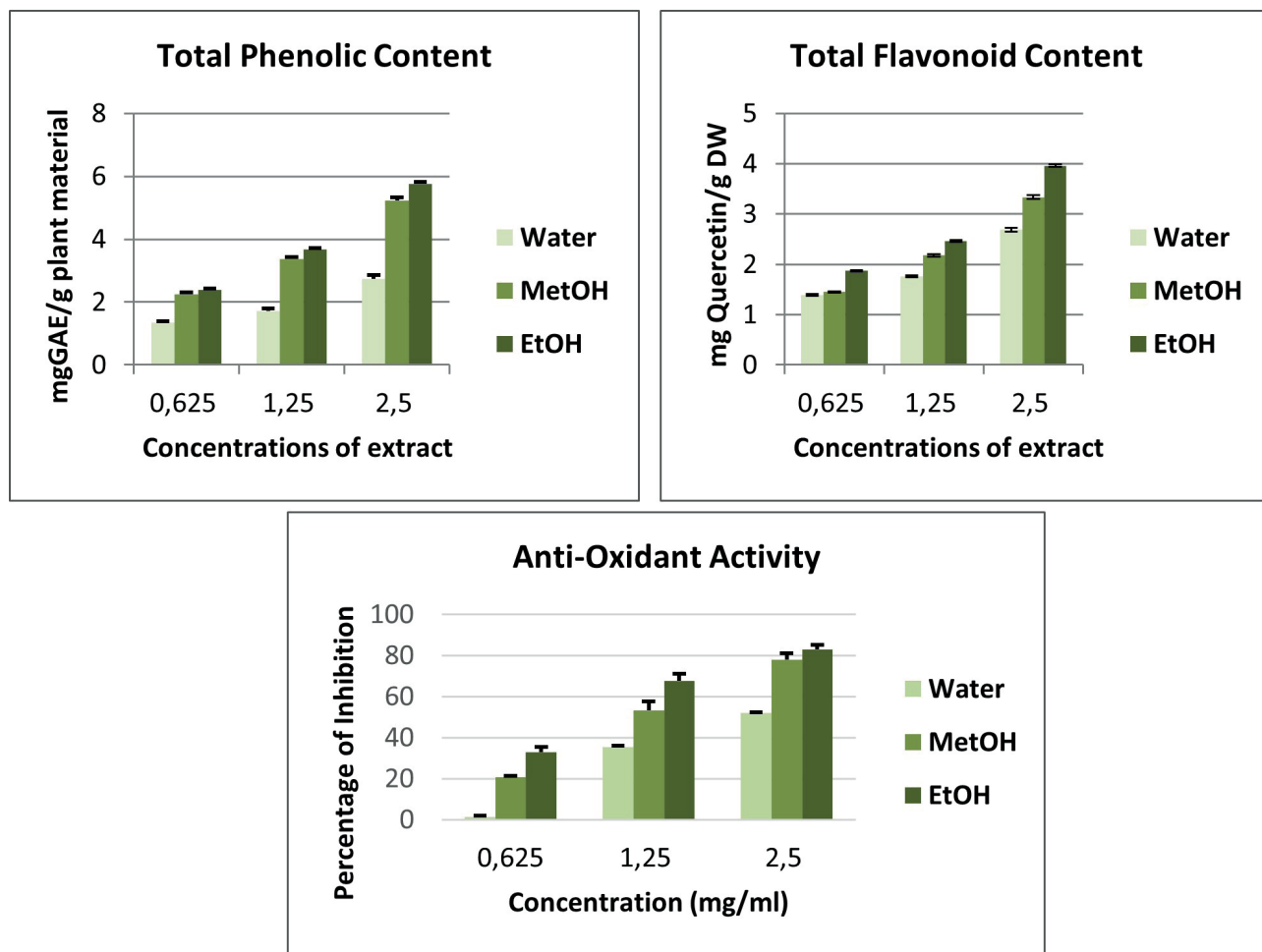


Figure 1. Comparison of phenol, flavonoid, and anti-oxidant analysis of ethanol, methanol, and water extracts showed that ethanol was the best solvent for extraction of *H. helix*.

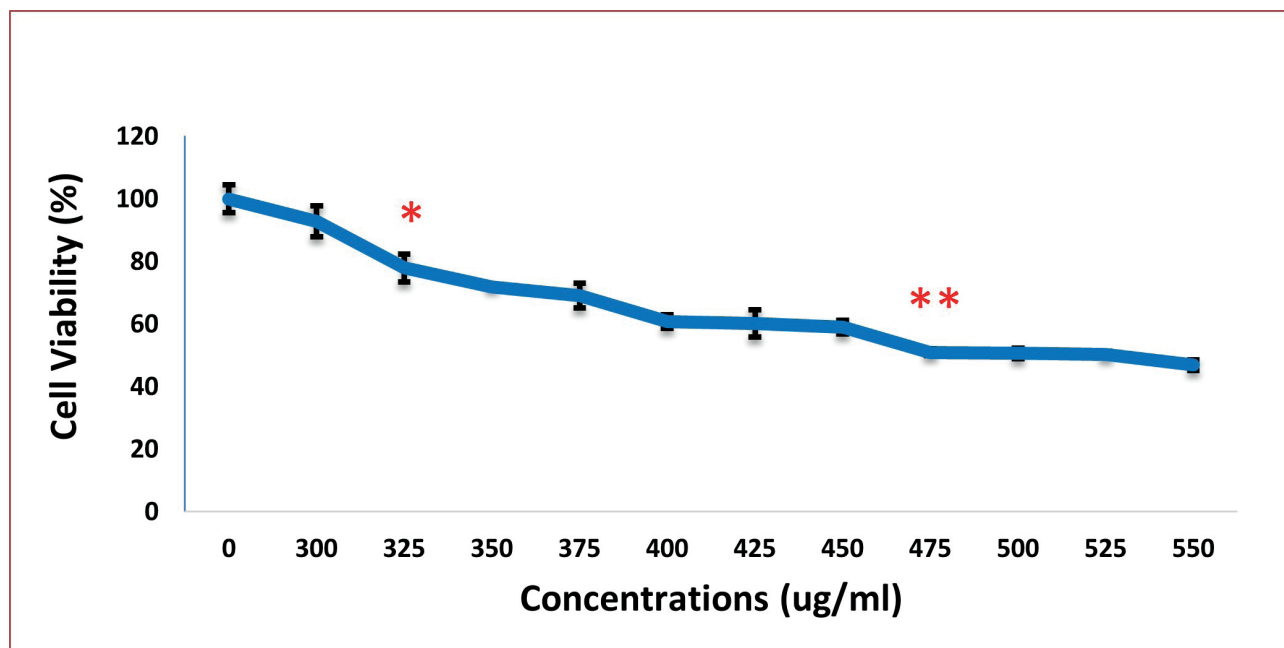


Figure 2. Viability test results of *H. helix* ethanol extract on glioblastoma cancer cells. The difference in cytotoxicity efficacy compared to the control was statistically significant from 325 µg/mL. Data are presented as mean ± SD. The significant difference compared to the control is indicated by *p < 0.05 and ** p < 0.01.

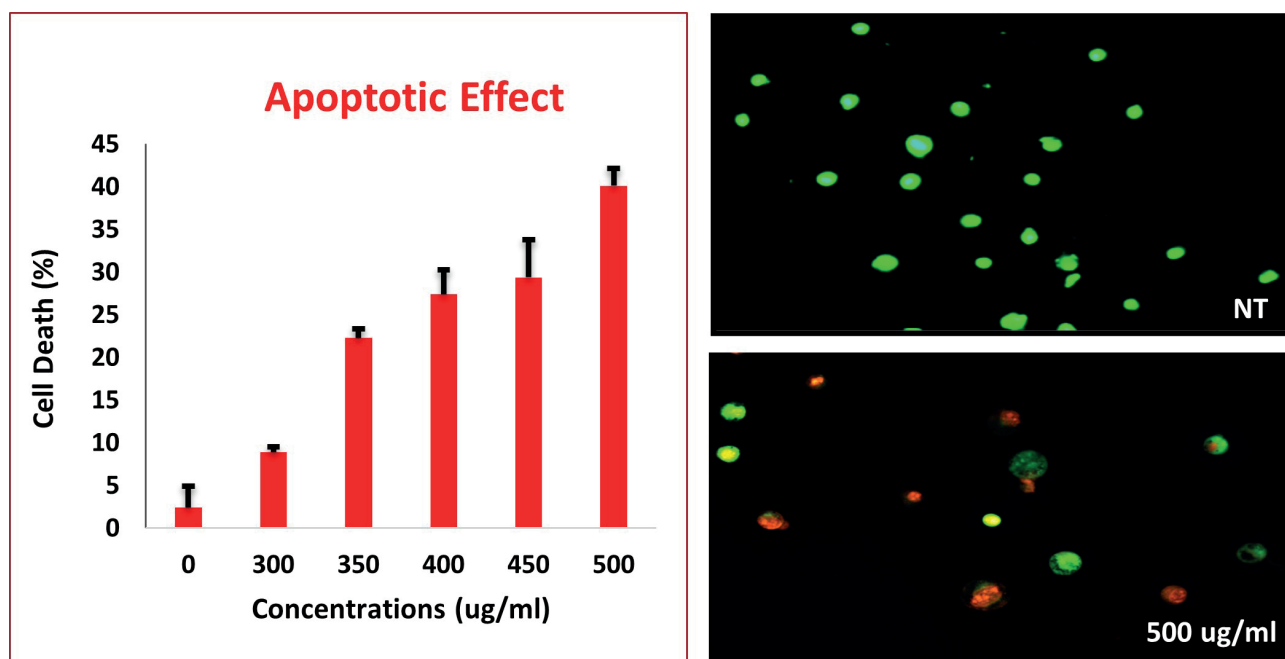


Figure 3. The cell death comparison of different concentrations of *H. helix* extract on glioblastoma cells was analyzed using AO/EB method. Dead cells (red) and live cells (green) were examined by fluorescence microscopy.

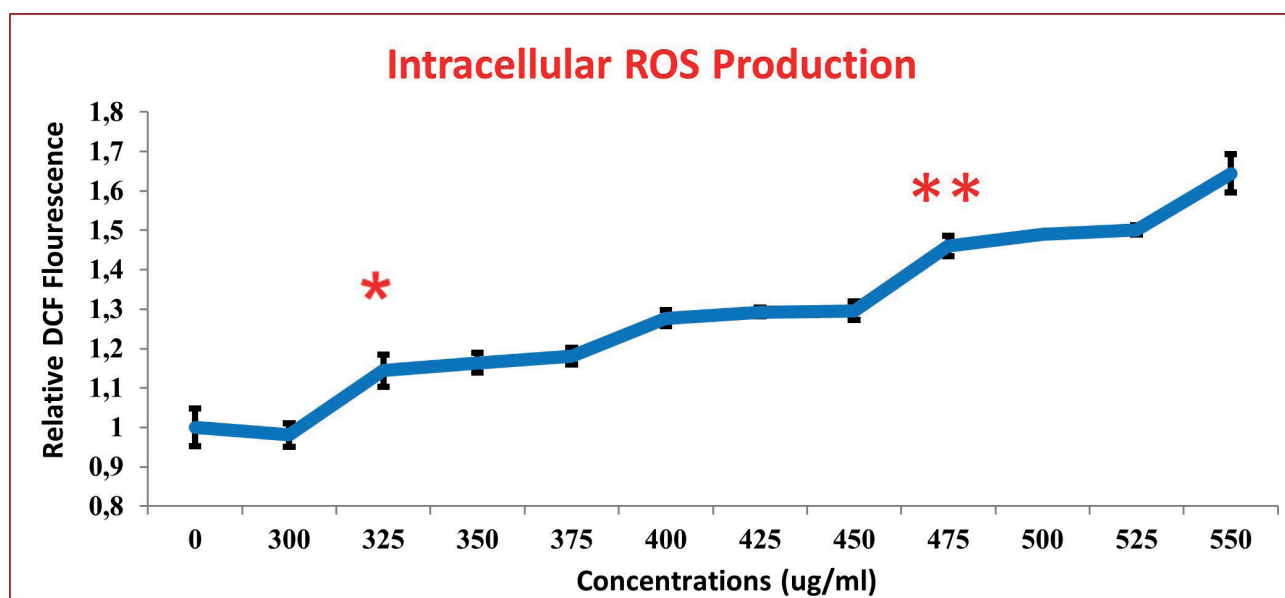


Figure 4. ROS generating effect of *H. helix* ethanol extract in glioblastoma cells. Extract started to increase ROS generation from the doses of 325 µg/ml. Results are presented as mean ± SD. The significant difference compared to the control is indicated by * $p < 0.05$ and ** $p < 0.01$.

in both developing and developed countries due to its natural origin and fewer reported side effects [25]. *H. helix*, commonly known as ivy leaves, is a plant that contains a diverse range of metabolites and has been traditionally used for treating the common cold, cough, and bronchial disorders [26]. In addition, ivy leaves have been explored as a potential alternative medicine for managing rheumatoid arthritis [26]. Although there are many studies on the therapeutic effects of *H. helix*, the best effect has been shown for reducing

the frequency and intensity of cough [27]. Rai demonstrated the anti-inflammatory properties of ivy leaves, where intraperitoneal injections of 7.5 ml/kg of ethanol extract resulted in 88.89% inhibition of formalin-induced paw edema in Swiss Albino mice. The anti-inflammatory activity of the ivy leaf extract was comparable to that of the reference drug diclofenac, which exhibited 94.44% inhibition [11]. In another study, the *H. helix* extract inhibited IL-6 and IL-8 secretion and blocked the VEGF pro-angiogenic factor after inflammation

was induced by lipopolysaccharide (LPS) to mimic the bacterial infection and TNF- α for the acute phase systemic inflammation [28]. According to a recent article by Shokry et al. (2022), extracts of ivy leaves may be a promising option for treating rheumatoid arthritis. The researchers proposed that the flavonoid content in ivy leaves may be responsible for their potential therapeutic effects. The study found that the ivy leaf extract was able to suppress biochemical, oxidative, and pathological changes associated with Adjuvant-induced arthritis [29]. Roşca-Casian also reported the antifungal activity of *H. helix* leaf ethanolic extract against phytopathogenic fungi [30].

In the current study, we primarily extracted *H. helix* leaves in three solvents; ethanol, methanol, and water, then compared these extracts for phenolic and flavonoid contents besides antioxidant activity since a positive correlation was shown between antioxidant effects, polyphenolic and flavonoid contents for *H. helix* ethanolic extract [12]. Our results showed that the ethanol and methanol extracts gave higher results for phenol and flavonoid contents together with antioxidant activity compared to the aqueous extract, and ethanol was the best among them. These results were also compatible with the literature that the hydro-alcoholic extract of *H. helix* showed better in vitro activity against adult parasites compared to the aqueous extract [13].

In literature, *H. helix* was also investigated for its antitumor effects besides all its pharmacological and therapeutic activities. *H. helix* ethanolic leaf extract was shown to suppress the migration of Mat-LyLu cells (strongly metastatic rat prostate cancer cells) together with proliferation and mitotic activity [15]. Rehman et al also showed that *H. helix* leaf extracts decrease cell viability on HT-29 colon cancer cells [14]. The previous studies revealed a lack of research on the potential anti-tumor effects of *H. helix* leaf extract on glioblastoma cells. The antitumor effects of Hederagenin, a triterpenoid acid abundant in ivy leaves, on glioma were first investigated by Dai et al., who observed that Hederagenin inhibited cell proliferation, invasion, and migration in U87 and U251 glioma cell lines [31]. In our current study, we investigated the effects of ethanolic extract of ivy leaves on U87 cells for the first time. Our results showed that the extract decreased cell viability and induced apoptosis.

Although *H. helix* has antioxidant phenolic compounds [8], it shows pro-oxidant activity and generates ROS due to the Fenton reaction in the presence of transition metals such as iron and copper [32]. While low levels of ROS play important roles in various cellular functions including signaling and regulation of gene expression [33], elevated levels of ROS can cause cellular damage, leading to cell death [34]. Excessive ROS can lead to a state of oxidative stress within cells, which can damage cellular components such as lipids, proteins, and DNA. This damage can trigger several mechanisms of cell death, including apoptosis, necrosis, and autophagy [35-37]. In this study, it was observed that the extract of *H. helix* increased intracellular ROS generation in U87 cells in a dose-dependent manner. This finding is consistent with previous literature, which demonstrated that α -Hederin, a monodesmosidic triterpenoid saponin isolated from *Hedera helix*, induces apoptosis and autophagic cell death in gastric [38] and colorectal cancers [39] through generating ROS.

To the best of our knowledge, this study is the first to investigate the effects of *H. helix* leaf ethanol extract on cytotoxicity and apoptosis in glioblastoma (U87) cells and to explore the relationship between these effects and ROS production. Its cytotoxic and apoptotic effect on glioblastoma cells may be due to its ROS-generating effect in a dose-dependent manner. While this study is intriguing and sheds light on the potential anti-cancer properties of *H. helix* on glioblastoma, this study focused on the U87 glioblastoma cell line, which represents only one type of cancer cell line. In addition, glial cells have not been examined for the effects of *H. helix* on normal cells. Further research is needed to address these limitations to show the potential anti-cancer effects of *Hedera helix* ethanol extract on glioblastoma.

CONCLUSION

According to the results obtained, *Hedera helix* ethanol extract was found to have dose-dependent cytotoxic and apoptotic effects in the U87 glioblastoma cell line. It has been concluded that high doses of *H. helix*, an antioxidant plant, may exhibit anti-cancer effects through pro-oxidant activity.

Author contribution

Study conception and design: AK; data collection: VBY, ED, FK; analysis and interpretation of results: AK and VBY; draft manuscript preparation: VBY and EK. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

This study does not require ethical approval since it does not contain any studies with human or animal subjects.

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Conflict of interest

The authors declare that there is no conflict of interest.

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