ARAŞTIRMA MAKALESİ / *RESEARCH ARTICLE*

Cytotoxic Effects of Helichrysum *arenarium* on HepG2 Cells

Helichrysum arenarium'un HepG2 Hücreleri Üzerindeki Sitotoksik Etkileri

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ABSTRACT

Aim: *Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide. The search for natural compounds with potential anticancer properties has gained increasing attention. Helichrysum arenarium is a medicinal plant known for its bioactive compounds, but its cytotoxic effects on liver cancer cells remain unexplored. This study aimed to evaluate the cytotoxic effects of Helichrysum arenarium extract on HepG2 liver cancer cells by assessing cell viability and morphological changes.*

Material and Method: *HepG2 cells were treated with different concentrations (0.025–0.1 mg/mL) of Helichrysum arenarium extract for 24 hours. Morphological alterations were observed using phase-contrast microscopy, while cell viability was assessed using the WST-1 assay. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test, considering p <0.05 as statistically significant.*

Results: *Helichrysum arenarium exhibited a dose-dependent cytotoxic effect on HepG2 cells. Morphological observations indicated apoptotic-like changes, and the WST-1 assay showed a significant decrease in cell viability, with the highest concentration (0.1 mg/mL) reducing viability to below 30% of the control (p <0.05).*

Conclusion: *These findings suggest that Helichrysum arenarium exhibits potent cytotoxic effects on HepG2 liver cancer cells, likely through potential apoptotic pathways. Further studies are needed to elucidate the molecular pathways involved and to evaluate its potential as a therapeutic agent for hepatocellular carcinoma.*

Key words: *Helichrysum arenarium; HepG2 cell line; hepatocellular carcinoma; cytotoxicity; medicinal plants*

ÖZET

Amaç: *Hepatoselüler karsinom (HCC), dünya genelinde kansere bağlı ölümlerin önde gelen nedenlerinden biridir. Potansiyel antikanser özelliklere sahip doğal bileşiklerin araştırılması giderek artan bir ilgi görmektedir. Helichrysum arenarium, biyoaktif bileşenleri ile bilinen tıbbi bir bitkidir, ancak karaciğer kanseri hücreleri üzerindeki sitotoksik etkileri henüz araştırılmamıştır. Bu çalışmanın amacı, Helichrysum arenarium ekstraktının HepG2 karaciğer kanseri hücreleri üzerindeki sitotoksik etkilerini hücre canlılığı ve morfolojik değişiklikler açısından değerlendirmektir.*

Materyal ve Metot: *HepG2 hücreleri, farklı konsantrasyonlarda (0,025–0,1 mg/mL) Helichrysum arenarium ekstraktı ile 24 saat süreyle muamele edilmiştir. Morfolojik değişiklikler faz-kontrast mikroskobu kullanılarak gözlemlenmiş, hücre canlılığı ise WST-1 testi ile değerlendirilmiştir. İstatistiksel analiz, tek yönlü ANOVA ve Tukey'nin post-hoc testi kullanılarak gerçekleştirilmiş olup, p <0,05 değeri istatistiksel olarak anlamlı kabul edilmiştir.*

Bulgular: *Helichrysum arenarium'un HepG2 hücreleri üzerinde doz-bağımlı sitotoksik bir etkisi olduğu gözlemlenmiştir. Morfolojik gözlemler apoptotik benzeri değişikliklere işaret etmiş ve WST-1 testi hücre canlılığında önemli bir azalma göstermiştir; en yüksek konsantrasyon (0,1 mg/mL) canlılığı kontrolün %30'unun altına düşürmüştür (p <0,05).*

Sonuç: *Bu bulgular, Helichrysum arenarium'un HepG2 karaciğer kanseri hücreleri üzerinde güçlü sitotoksik etkiler gösterdiğini ve bunun muhtemelen apoptozla ilişkili mekanizmalar yoluyla gerçekleştiğini düşündürmektedir. Moleküler yolların aydınlatılması ve hepatoselüler karsinom için potansiyel bir terapötik ajan olarak değerlendirilmesi için ileri çalışmalara ihtiyaç vardır.*

Anahtar kelimeler: *Helichrysum arenarium; HepG2 hücre hattı; hepatoselüler karsinom; sitotoksisite; tıbbi bitkiler*

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Introduction

Hepatocellular carcinoma (HCC) is the most prevalent subtype of liver cancer, accounting for approximately 75% of all cases. It ranks as the fourth leading cause of cancer-related mortality worldwide. Due to its aggressive nature, HCC is often diagnosed at an advanced stage, and prognosis is particularly poor in patients with pre-existing liver dysfunction[1]. Hepatoma cells are widely utilized as models to study both the phenotypic characteristics of liver cancer and the behavior of hepatocytes under healthy and diseased conditions. Among human hepatoma cell lines, HepG2 cells are the most extensively characterized. Their versatility and functional properties make them invaluable for investigating liver functions[2].

Helichrysum arenarium (L.) Moench, commonly known as "sandy everlasting", is a perennial plant of the *Asteraceae* family[3]. Native to Europe, it is also found in Türkiye, particularly in the Western Black Sea, Central Anatolia, and Eastern Anatolia regions. Traditionally, the plant has been used in European ethnomedicine for its hepatoprotective, bile-stimulating, and detoxifying properties[4]. Its inflorescences are rich in flavonoids (e.g., chalcone isosalipurposide, naringenin), essential oils, carotenoids, and α-pyrone derivatives[5,6].

Türkiye's diverse climate and rich flora support the growth of various *Helichrysum* species, which are commonly consumed as herbal teas for their bile-regulating and diuretic properties[7]. Due to the documented pharmacological properties of *Helichrysum arenarium*, this study aims to evaluate its cytotoxic effects on HepG2 liver cancer cells in terms of cell viability and morphological alterations. The hypothesis of this study is that *H. arenarium* extract may induce apoptosis-related cytotoxicity in HepG2 cells.

Materials And Methods

Collection and Authentication of Plant Material

Fresh *Helichrysum arenarium* samples were collected from the Giresun region of Türkiye. The plant material was authenticated by a botanist, and voucher specimens were deposited for future reference. The collected samples were stored under control conditions until further processing.

Preparation of Plant Extracts

The extraction process was performed using dried *Helichrysum arenarium*. A total of 10 mg of dried plant material was weighed and placed into a sterile container. The extraction solution, preheated to 65°C, was added, and the mixture was transferred to a 50 mL Falcon tube. The Falcon tube containing the dried herb was frozen in liquid nitrogen and mechanically crushed while maintaining an air-tight environment. The crushed sample was then incubated at 65°C for 15 minutes. Following incubation, chloroform (Sigma Aldrich, USA) at a 24:1 v/v ratio was added to the Falcon tube and thoroughly mixed. The sample was centrifuged at 2000 × g for 15 minutes at room temperature, after which the upper liquid phase was carefully transferred to a new Falcon tube. The extraction process was repeated with an additional 24:1 v/v chloroform treatment, followed by another 15-minute centrifugation at 2000 × g at room temperature. The resultant liquid phase was combined with 6 mL of isopropanol (Sigma Aldrich, USA) and incubated at -20°C for 5 minutes. The final step involved centrifugation at 8500 × g for 10 minutes, and the supernatant was transferred to a new Falcon tube. The sample was left to rest for three days before being washed twice with 70% ethanol in a water bath maintained at 65°C to ensure purity[8].

Cell Culture

HepG2 cells, obtained from the laboratories of Istanbul University, were maintained under standard culture conditions. Firstly, the liquid nutrient medium was removed, and the cells were washed with 5 mL of phosphate-buffered saline (PBS; Gibco). Following the PBS wash, 1.2 mL of Trypsin (Gibco) was added to facilitate cell detachment, and the cells were incubated at 37°C for 1–2 minutes to activate Trypsin.

Upon confirmation of cell detachment from the culture dish, 5 mL of Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) was added to neutralize Trypsin activity. The cell suspension was then transferred into a 15 mL Falcon tube and centrifuged at 2000 rpm for 5 minutes. Following centrifugation, the supernatant was discarded, and the cell pellet was resuspended in 5 mL of DMEM supplemented with 10% FBS. The centrifugation step was repeated under the same conditions to ensure complete removal of Trypsin.

The cell pellet was resuspended in 2 mL of DMEM, and the viable cell count was determined using a hemocytometer and Trypan Blue staining (Sigma Aldrich). Subsequently, cells were seeded into a 96-well plate at a density of 1×10^4 cells per well. The cultures were incubated for 24 hours under standard conditions. Following incubation, cell morphology was observed and documented using a phase-contrast microscope.

In addition to untreated control wells, a solvent control group was included to assess the cytotoxicity of the extraction solvents. The solvent control group received a mixture of chloroform and isopropanol at the same volume and ratio used in the extract preparation. This group was subjected to the same incubation conditions and cell viability was assessed using the WST-1 assay.

Detection of Morphological Changes

HepG2 cells were exposed to different concentrations (0.025–0.1 mg/mL) of *Helichrysum arenarium* extract for 24 h. After the exposure period, cells were observed under an inverted light microscope to detect any morphological changes related to apoptosis.

Analysis of Cell Viability

Cell viability was assessed using the WST-1 colorimetric assay after 24 hours of treatment with different concentrations of *Helichrysum arenarium* extract. Each plate contained blanks, controls, and dilution series with three replicates. The WST-1 assay relies on the cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenases, with the intensity of the resulting dark yellow formazan dye directly correlating to cell viability. After incubation, a 10 µl aliquot of WST-1 reagent was added to each well (for a total 100 µl solution, maintaining a 9:1 ratio) and further incubated at 37°C for 2 hours. Absorbance was measured at 450 nm using a Multiskan SkyHigh Reader (Thermo Fisher Scientific, USA), with higher absorbance values indicating greater cell viability[9–11].

Statistical Analysis

The data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The results are presented as mean ± standard deviation (SD). One-way ANOVA was used to assess statistical differences between groups, followed by Tukey's post-hoc test for multiple comparisons. The significance level of p <0.05 was considered statistically significant.

Results

Morphological Changes in HepG2 Cells

HepG2 cells were treated with *Helichrysum arenarium* extract at concentrations ranging from 0.025 mg/mL to 0.1 mg/mL for 24 hours (Fig. 2). Microscopic analysis revealed distinct morphological changes in a dose-dependent manner. In control conditions, cells maintained a typical epithelial-like morphology with well-defined cell membranes and strong adherence to the culture plate. However, cells exposed to increasing concentrations of the extract exhibited features consistent with cytotoxicity, including reduced cell density, cell shrinkage, membrane blebbing, and detachment from the surface. The most pronounced effects were observed at 0.075 mg/mL and 0.1 mg/mL, where a significant proportion of cells became rounded and detached, indicative of apoptosis. The solvent control group did not show any significant decrease in cell viability compared to the untreated control, indicating that the observed cytotoxic effects were specific to the plant extract.

WST-1 Cell Viability Assay

The WST-1 assay results further confirmed the cytotoxic effect of Helichrysum arenarium on HepG2 cells. A concentration-dependent decrease in cell viability was observed (Fig. 1a). The absorbance values presented in Fig. 1b numerically support this trend, showing a marked decline with increasing extract concentrations. At 0.025 mg/mL, there was a slight but statistically significant reduction in viability (p <0.05). At 0.05 mg/mL and higher, a marked decrease in absorbance values indicated a strong cytotoxic response. The highest tested concentration (0.1 mg/mL) reduced to below 30% of control values. The statistical analysis confirmed that all treatment groups, except the lowest concentration, significantly differed from the control (p <0.05, Tukey's test). These findings suggest that *Helichrysum arenarium* disrupts mitochondrial activity and inhibits cell proliferation in HepG2 cells. The solvent control group exhibited no statistically significant difference in cell viability compared to the untreated control group (p >0.05), indicating that the observed cytotoxic effects were specific to *Helichrysum arenarium* extract and not due to the solvents used in the extraction process. In addition to the graphical data, tabulated absorbance values (Fig. 1b) provide numerical support for the dose-dependent decrease in viability, showing a decline in mitochondrial activity at increasing extract concentrations.

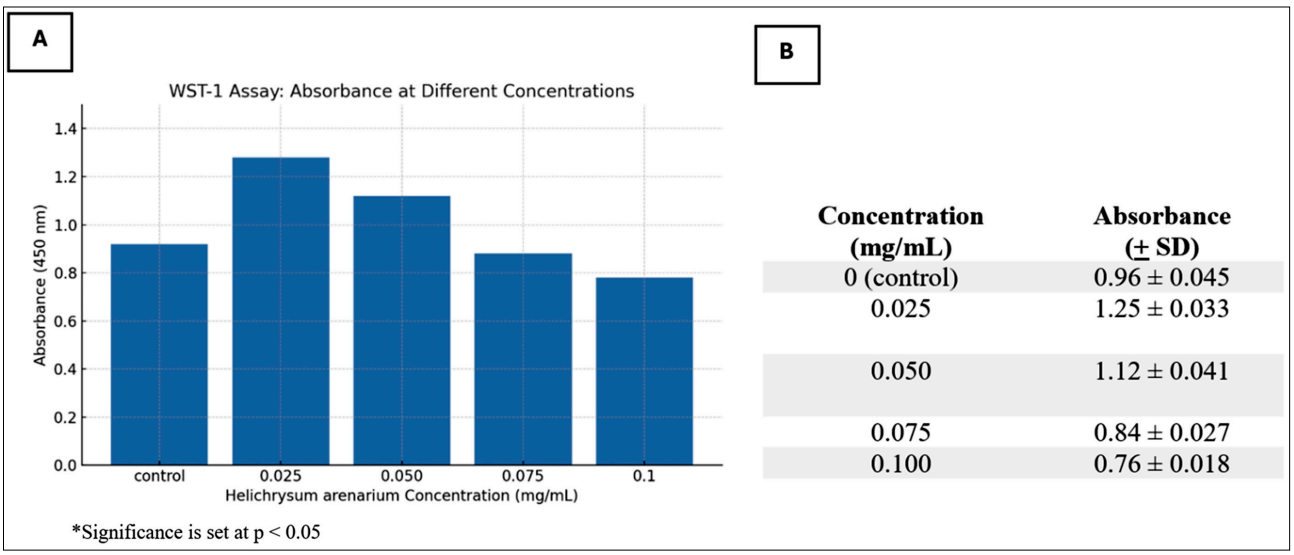

Figure 1. *Evaluation of Helichrysum arenarium's effect on HepG2 cells with WST-1 assay.*
(A) Bar graph showing the absorbance values at 450 nm, indicating mitochondrial activity in HepG2 cells after 24-hour treatment with different concentrations of Helichrysum arenarium extract. Interestingly, a slight increase in absorbance is observed at 0.025 mg/mL, which may suggest a transient proliferative or metabolic activation effect. However, higher concentrations resulted in a marked, dose-dependent decrease in absorbance, reflecting reduced cell viability. (B) Table summarizing the absorbance values (mean ± SD) for each concentration. The consistency between graphical and tabular data supports the conclusion that Helichrysum arenarium exhibits concentration-dependent cytotoxic effects.

Discussion

Hepatocellular carcinoma (HCC) poses a growing global health challenge, with its incidence and mortality rates steadily rising[12]. According to estimates from the World Health Organization, liver cancer-related deaths are expected to exceed 1 million by 203012.

The search for natural compounds with anticancer properties has gained attention, with medicinal plants emerging as potential therapeutic agents. This study evaluated the cytotoxic effects of *Helichrysum arenarium* extract on HepG2 liver cancer cells, demonstrating significant dose-dependent inhibition of cell viability and morphological alterations indicative of apoptosis[13–15]. Furthermore, the tabular data in Fig. 1b substantiates the visual evidence from microscopy and WST-1 bar graphs, clearly illustrating the progressive loss of mitochondrial activity with rising extract doses. While such morphological changes are consistent with apoptosis, the observed pattern in this study –particularly the rounded, detached cells at higher concentrations indicates early apoptotic features specific to HepG2 response under Helichrysum arenarium exposure.

The morphological changes observed under phase-contrast microscopy align with typical apoptotic features, including cell shrinkage, detachment, and membrane blebbing. These findings suggest that *Helichrysum arenarium* induces programmed cell death in HepG2 cells, which may be mediated through mitochondrial dysfunction and oxidative stress. Similar effects have been reported for other flavonoid-rich plant extracts, supporting the hypothesis that polyphenols present in *Helichrysum arenarium* contribute to its cytotoxic activity[3]. In this study, however, it should be noted that the apoptosis claim is based solely on morphological observations and WST-1 metabolic activity. No direct biochemical validation (such as caspase-3 activation, Annexin-V staining, or TUNEL assay) was performed, which limits the strength of the mechanistic conclusion.

The WST-1 assay results further corroborate the cytotoxic potential of *Helichrysum arenarium*. The significant reduction in metabolic activity at concentrations above 0.05 mg/mL indicates that the extract interferes with mitochondrial dehydrogenase enzymes, leading to decreased ATP production and cell death. Previous studies have shown that flavonoid-containing plant extracts can modulate apoptosis-related signaling pathways in various cancer cell lines. The active compounds in *Helichrysum arenarium*, including flavonoids and essential oils, may exert their effects through similar mechanisms[16].

While our findings demonstrate promising anticancer activity, there are limitations to this study. The experiments were conducted in an *in vitro* setting, which does

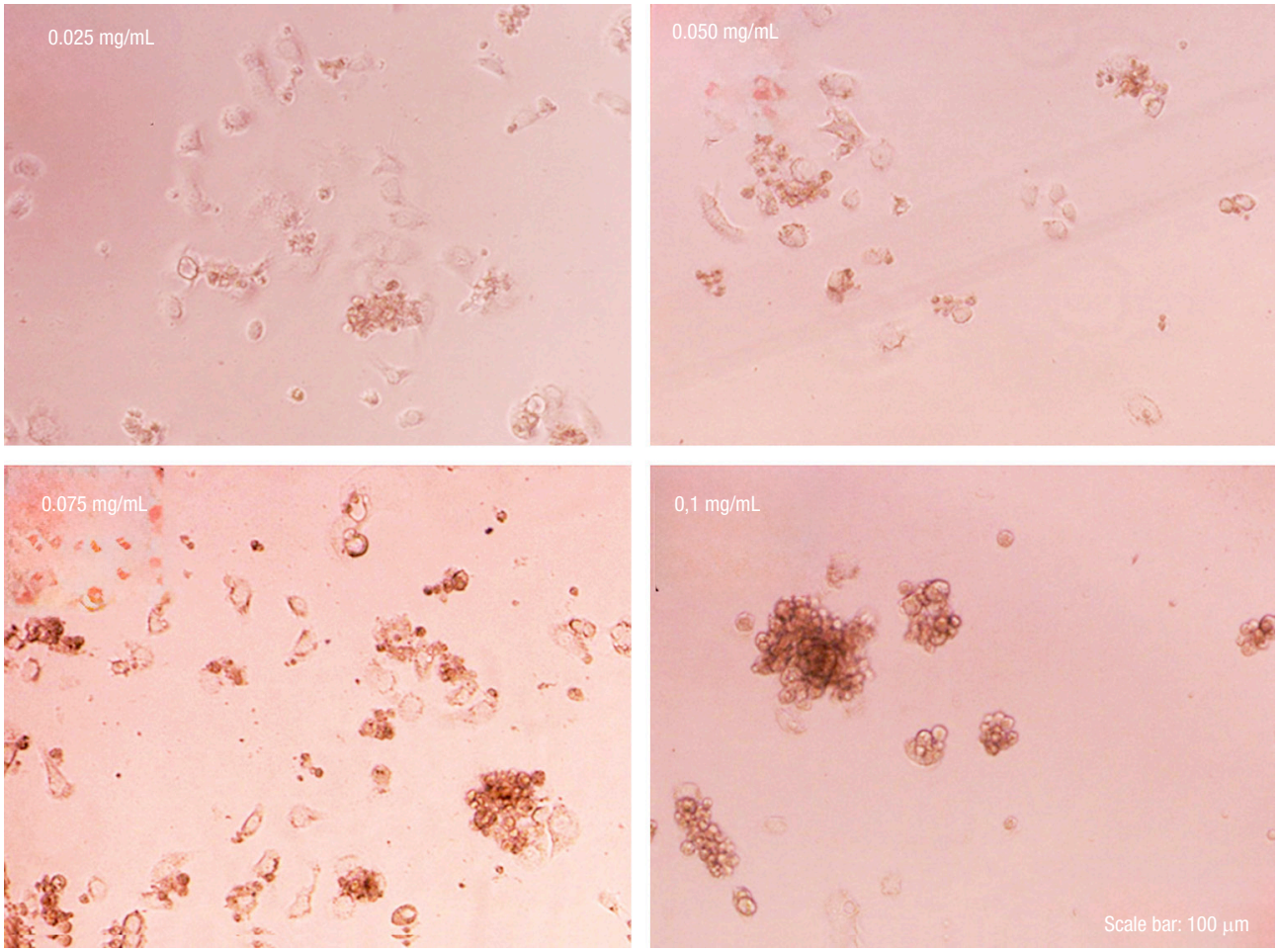

Figure 2. *Morphological changes in HepG2 cells after 24-hour treatment with Helichrysum arenarium at different concentrations, showing dose-dependent cytotoxic effects.*

not fully replicate the complexity of tumor microenvironments in vivo. Future studies should focus on elucidating the molecular mechanisms underlying the cytotoxic effects of *Helichrysum arenarium* and assessing its efficacy in animal models. Additionally, identifying the specific bioactive compounds responsible for its anticancer properties could provide valuable insights for the development of novel chemotherapeutic agents. The observed correlation between morphological alterations and WST-1 cell viability results enhances the robustness of the findings and reinforces the hypothesis that *Helichrysum arenarium* exerts its cytotoxic effects through potential apoptotic pathways. To further validate these in vitro results, future studies could incorporate in vivo xenograft models –such as HepG2-derived hepatocellular carcinoma tumors in athymic nude mice– to comprehensively assess the therapeutic potential, pharmacokinetics, and bioavailability of the extract.

Conclusion

This study demonstrated that Helichrysum arenarium extract exerts significant cytotoxic effects on HepG2 liver cancer cells, reducing cell viability and inducing morphological features associated with apoptosis. While the findings are promising, the study was limited to *in vitro* conditions. Future research should focus on identifying the active constituents responsible for these effects, elucidating the underlying molecular mechanisms, and validating the extract's therapeutic potential through *in vivo* studies. These efforts could contribute to the development of novel plant-based agents for the treatment of hepatocellular carcinoma.

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This article has not benefited from any financial resources.

Conflict of Interest

There is no conflict of interest related to this article.

Ethical Approval

This study was conducted using commercially available ready-to-use cell cultures and, therefore, does not require ethical approval.

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