



In Vitro Cytotoxicity and Oxidative Stress Evaluation of Valerian (*Valeriana officinalis*) Methanolic Extract in Hepg2 and Caco2 Cells

Valeriana officinalis'in Metanol ile Hazırlanmış Ekstresinin HepG2 ve Caco2 Hücrelerinde *In Vitro* Sitotoksiste ve Oksidatif Stres Değerlendirmesi

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ABSTRACT

Objectives: Traditional treatment methods are becoming popular and commonly used in many societies and have become the first treatment option for most people. While some of these methods are helpful, they can interact with medications the patient is taking for another disease and cause a variety of life-threatening risks. Valerian (catweed) plant is used in traditional medicine as a sleep aid due to its sedative effects. Valerian may also exert anticancer effect *in vitro*.

Materials and Methods: In this study, the cytotoxicity and oxidative stress effects of valerian root extract were evaluated in human liver hepatocellular carcinoma (HepG2) and human colorectal adenocarcinoma (Caco2) cell lines. The cytotoxicity was evaluated via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test. Total reactive oxygen species analysis was performed via a 2',7'-dichlorodihydrofluorescein diacetate assay in flow cytometry.

Results: Inhibition concentration 50 values were calculated as 936.6 and 1097.5 µg/mL in the HepG2 and Caco2 cell lines, respectively. It was observed that valerian root extract did not induce oxidative stress in HepG2 and Caco2 cell lines.

Conclusion: These results indicate that the use of valerian root extract as an alternative method in cancer treatment may not be effective and may cause a risk for public health. On the other hand, it may be safe at recommended tolerated concentrations since it does not cause oxidative stress.

Key words: *Valeriana officinalis*, HepG2, Caco2, oxidative stress, MTT

ÖZ

Amaç: Geleneksel tedavi yöntemleri, birçok toplumda yaygın olarak kullanılmakta, popüler hale gelmekte ve birçok kişi için ilk tedavi seçeneği olarak karşımıza çıkmaktadır. Bu yöntemlerden bazıları yararlı olmakla birlikte, kişinin başka bir hastalık için kullandığı ilaçlarla etkileşime girebilir ve çeşitli yaşamı tehdit edici risklere neden olabilir. Kediotu (catweed) bitkisi yatıştırıcı etkisi nedeniyle geleneksel tıp uygulamalarında uyku düzenleyici amaçlı kullanılmaktadır. Ayrıca *in vitro* olarak kanser önleyici etkiye sahip olabileceği bildirilmiştir.

Gereç ve Yöntemler: Bu çalışmada, kediotu kökü ekstresinin sitotoksiste ve oksidatif stres etkileri HepG2 ve Caco2 hücre hatlarında değerlendirilmiştir. Sitotoksiste değerlendirilmesi 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide testi ile gerçekleştirildi. Total-reaktif oksijen bileşikler analizi, hücre akış sitometrisinde 2',7'-dichlorodihydrofluorescein diacetate testi ile gerçekleştirildi.

Bulgular: İnhibisyon konsantrasyonu 50 değerleri HepG2'de 936,6 µg/mL ve Caco2 hücre hatlarında 1097,5 µg/mL olarak hesaplandı. Kediotu kökü ekstresinin HepG2 ve Caco2 hücre hatlarında da oksidatif strese neden olmadığı gözlemlenmiştir.

Sonuç: Bu sonuçlar, kanser tedavisinde alternatif bir yöntem olarak kediotu kökü ekstresi kullanımının etkili olamayacağını ve halk sağlığı açısından risk oluşturabileceğini, diğer yandan oksidatif strese neden olmadığı için tavsiye edilen tolere edilen konsantrasyonlarda güvenli olabileceğini göstermektedir.

Anahtar kelimeler: *Valeriana officinalis*, HepG2, Caco2, oksidatif stres, MTT

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INTRODUCTION

The use of herbal products for the treatment of several diseases has been widespread from the past to the present. In developed countries, herbal remedies are considered over-the-counter drugs with strict regulations. However, in developing countries, the use of herbal therapy methods and products lacks control. Most of herbal drugs in the market have not been fully evaluated from the toxicological aspect; thus, these products may cause several adverse effects during therapy of the different body systems.¹

Valeriana belongs to Valerianaceae family, which includes approximately 300 species. *Valeriana officinalis* has been generally used as an alternative traditional medicine for insomnia and depression therapy. This medical plant has sedative, anticonvulsant, hypnotic, and anxiolytic effects. In addition, this plant has treatment potential for gastrointestinal and urinary system problems. The *V. officinalis* compounds obtained by extraction methods (EMs) include flavonoids, monoterpenes, sesquiterpenes, valepotriates, iridoids, alkaloids, acid-like gamma-aminobutyric acid (GABA), glutamine, and lignans, which affect the central nervous system and have antioxidant and vasorelaxant effects. Given these effects, *V. officinalis* is a popular herbal remedy choice to cure insomnia, headache, gastrointestinal system, cardiovascular system, and urinary tract problems.²⁻⁵

Valeriana herbal remedy can be effective in cancer patients. Valerian herb ingredients with anticancer effects have been given great concern by the scientific area. However, herbal therapy implementers have been attempting to cure cancer patients with insufficient valerian products.⁶

In this work, we have observed the cytotoxic and oxidative stress-inducing effects, which play important roles in killing cancer cells, of methanolic extract of *V. officinalis* on HepG2 and CaCo2 cell lines.

MATERIALS AND METHODS

Valeriana officinalis extraction

V. officinalis roots were purchased commercially from a traditional herbal drug store in Istanbul and pulverized in a porcelain mortar. A total of 75 mL methanol was added to 15 g powdered *V. officinalis* roots and incubated at room temperature in a shaker for 24 h. After the incubation period, the extraction solution was filtered with a Whatman no.1 filter, and methanol was evaporated with fractional distillation.⁷

Cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test human liver hepatocellular carcinoma (HepG2- HB-8065™) and human colorectal adenocarcinoma (Caco2- HTB-37™) cell lines were purchased from the American Type Culture Collection (Virginia, USA), and the cells were maintained as per manufacturer's instructions. Exactly 1000 mg/mL extract was prepared by dissolving *V. officinalis* roots in 100% dimethyl sulfoxide (DMSO) and stored at +4°C until the experiments. Before the cell treatments, the extract was diluted with DMSO at a final

concentration of 1%. Given the selected concentrations for cytotoxicity assay, the root extract was dissolved in a cell culture medium to prepare the desired concentrations. The treatments were performed at a concentration range for 24 h to evaluate the dose-dependent effects. All study experiments were performed in triplicates in three different days.

The HepG2 and Caco2 cells were seeded into 96-well plates (1x10⁴ cells/100 µL cell culture medium/well). After overnight incubation, the cells were treated with *V. officinalis* extract at the concentrations of 200, 400, 600, 800, and 1000 µM and control for 24 h. In the control group, the final DMSO concentration was 1%. Then, MTT was added into the wells, and the wells were incubated for another 3 h at 37°C in the dark. Optical densities were measured at 570 nm using a microplate reader (Biotek, Epoch, Vermont, USA).⁸

Total reactive oxygen species (Total-ROS) assay with 2',7'-dichlorodihydrofluorescein diacetate (H₂DC-FDA).

Different pathological conditions were associated with the ROS increase in cells. Thus, changes in the ROS level are detected in basic studies. Given the short half-life of ROS, effective detection methods are important during observations. H₂DC-FDA is a non-fluorescent dye, and in the presence of ROS, it returns a green fluorescent with oxidation.⁹ In the present study, the ROS production was evaluated with H₂DC-FDA dye with by a flow cytometer. The 5x10⁵ HepG2 and Caco2 cells in 2 mL medium per well were seeded into six-well plates and incubated overnight. The cells were treated with 100, 200, 100, and 600 µg/mL concentrations. These concentrations exerted a cell viability higher than 70% in the MTT assay for 24 h. The 1% DMSO solution was used as the negative control for the experiment. After 24 h, the cells were washed with phosphate-buffered saline (PBS) twice and incubated with 20 µM H₂DC-FDA at 37°C for 30 min. The cells were detached with trypsin-ethylenediaminetetraacetic acid after the incubation period and washed with PBS. Then, the cells were re-suspended with 1% BSA in 150 µL PBS. The fluorescence intensity of 10⁴ cells was measured with an ACEA NovoCyte flow cytometer (San Diego, California, USA), and the results were expressed as the percentage of median fluorescence intensity (MFI %) as previously described.¹⁰

Statistical analysis

All the experiments were performed as three replicates; the results were presented as the mean ± standard deviation. The statistical comparison results were analyzed using the one-way analysis of variance followed by Tukey's test for post hoc analysis, and the statistical significance was set at p<0.05 (SPSS, version 21.0, USA)

RESULTS

Cell viability

According to MTT results, the percentage of cell inhibition in Caco2 and HepG2 cell lines with *V. officinalis* methanolic extract exposure increased and was concentration dependent for 24 h (Figure 1, Table 1). The inhibition concentration 50 (IC₅₀) values

were calculated by graph slope formulations. The IC_{50} values were 939.68 $\mu\text{g/mL}$ for HepG2 cells and 1097.58 $\mu\text{g/mL}$ for Caco2 cells.

Total-ROS induction

The IC_{30} values of *V. officinalis* methanolic extract in HepG2 and Caco2 cell lines were calculated for the Total-ROS evaluation. The calculated IC_{30} values were 600.12 $\mu\text{g/mL}$ for HepG2 cells and 672.95 $\mu\text{g/mL}$ for CaCo2 cells. The 100, 200, 400, and 600 $\mu\text{g/mL}$ concentrations were selected for the Total-ROS analysis to determine the ROS production with a flow cytometer via $H_2DC\text{-FDA}$. No statistically significant difference was observed between the concentration groups for MFI for both cell lines. The *V. officinalis* methanolic extract did not cause an increase in the ROS production after a 24 h exposure (Figure 2).

DISCUSSION

Valerian belongs to Valerianaceae family, which includes approximately 300 species existing only in Western countries, and contains several different phytochemicals that may have nervous system protection, diuretic, antispasmodic, anthelmintic, antioxidant, antimicrobial, anti-inflammatory, antirheumatic sedative, anticonvulsant, and diaphoretic effects.⁶ As a medicinal herbal plant, valerian is important in traditional therapy for sleeping and anxiety disorders due to its effects on the GABA A receptor system.¹¹ *V. officinalis* products

are widely used for different type of diseases. In recent years, the usage of valerian products in cancer cure increased due to herbal implementers.²

Several people die worldwide due to different cancer types, and new therapeutic development studies pique the interest of scientists. Oxidative stress induction plays an important role in cancer cell death induction via chemotherapeutics. Oxidative stress has evident effects on cancer cell induction and cancer cell death mechanisms. Oxidative stress may induce cancer cell proliferation via DNA damage or can be a therapeutic strategy to cure cancer via inducing cancer cell death through

Table 1. MTT results of *Valeriana officinalis* extract exposure

Concentration ($\mu\text{g/mL}$)	Cell viability inhibition (%)	
	HepG2	Caco2
200	4.99	6.81
400	18.36	17.30
600	33.36	29.28
800	40.15	33.71
1000	52.95	45.72

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, HepG2: Human liver hepatocellular carcinoma, Caco2: Human colorectal adenocarcinoma

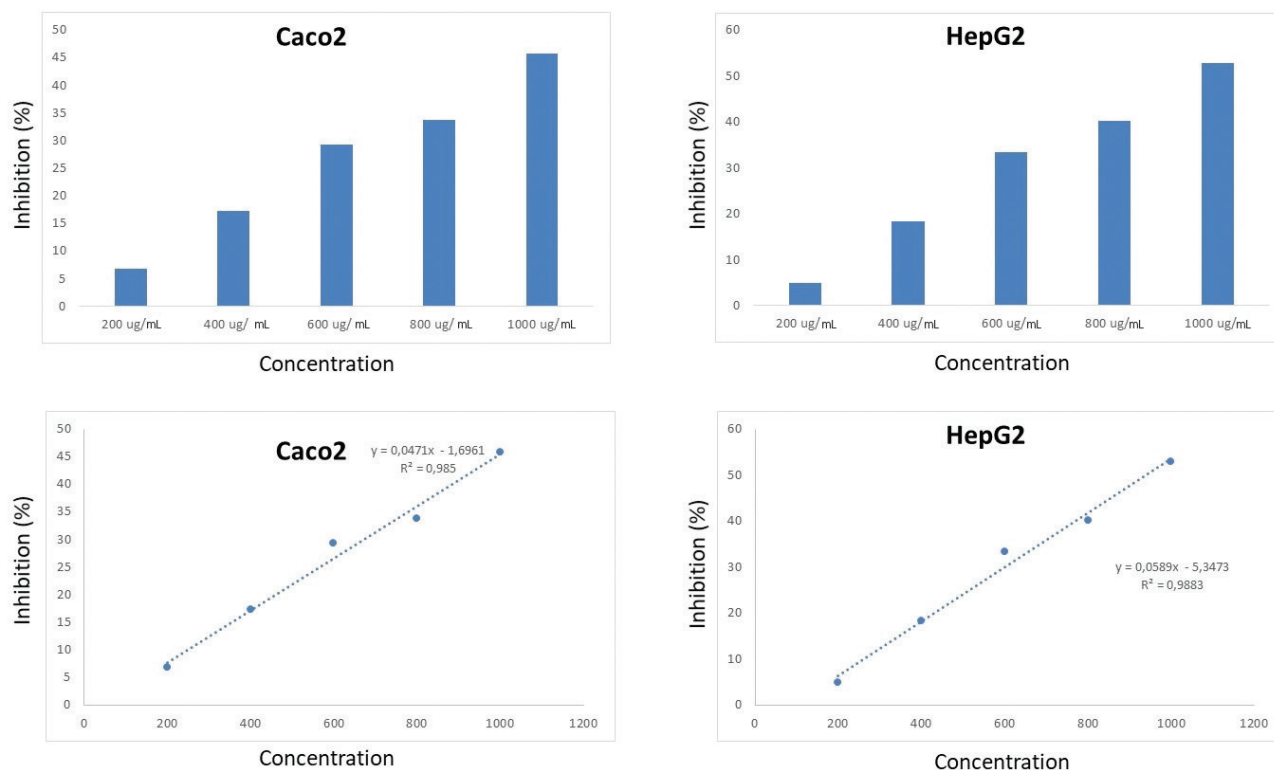


Figure 1. Cell viability inhibition (%) values of *Valeriana officinalis* methanolic extract on HepG2 and Caco2 cell lines increased with concentration dependence. Graph slope formulations are shown on the graphs

HepG2: Human liver hepatocellular carcinoma, Caco2: Human colorectal adenocarcinoma

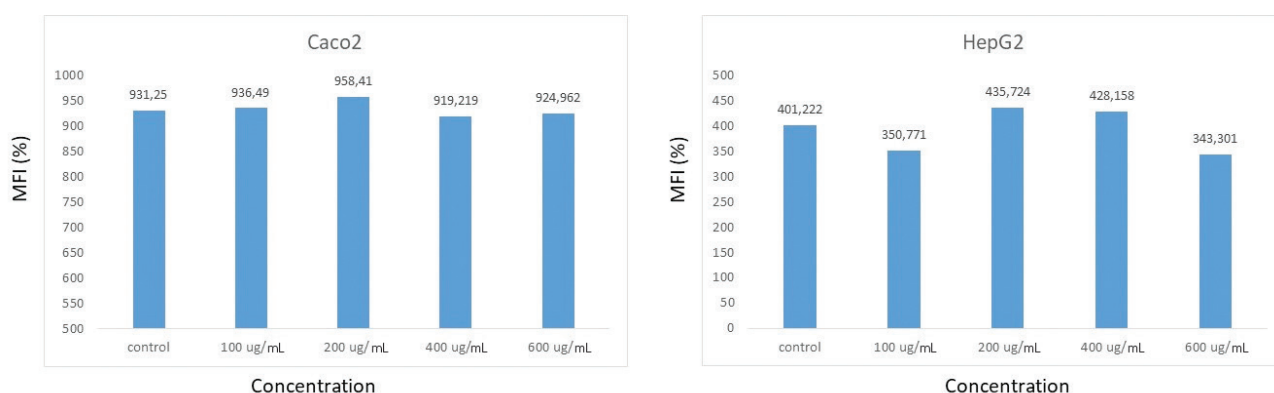


Figure 2. DC-FDA analysis results for HepG2 and Caco2 cell lines after 24 h exposure to *Valeriana officinalis* methanolic extract. ROS production was not induced ($p > 0.05$)

DC-FDA: 2',7'-dichlorodihydrofluorescein diacetate, HepG2: Human liver hepatocellular carcinoma, Caco2: Human colorectal adenocarcinoma, ROS: Reactive oxygen species

apoptosis.^{12,13} In different clinical trials, varied results were reported on valerian species *in vitro* and *in vivo*. In human neuroblastoma (SH-SY5Y) cell line, de Oliveria et al.² reported that aqueous *V. officinalis* extract exerted a protective role against apoptosis with rotenone exposure. Wang et al.¹³ showed the antioxidant effects of *V. jatamansi* on HepG2, human cervix carcinoma, breast cancer (MDA-MB-231), and human umbilical vein endothelial cells. Kakehashi et al.¹¹ reported that *V. sitchensis* added in drinking water of male F344 rats significantly suppressed the 8-hydroxy-29-deoxyguanosine generation after hepatocarcinogenesis initiation by diethylnitrosamine at 50, 500, and 5000 ppm concentrations. The antioxidant catalase levels and apoptosis increased, and cell proliferation significantly decreased. The apoptosis induction was associated with the suppression of c-myc, MafB, and cyclin D1 expression and increased expression levels of p21Waf1/Cip1, p53, and Bax. These results indicated that *V. sitchensis* administration can be associated with carcinogenesis inhibition.¹¹ Zhu et al.¹⁴ showed the anticancer effects of *V. jatamansi* F3 fraction *in vitro* and *in vivo*. In MCF-10A cells, the F3 fraction administration increased the intracellular ROS production and induced apoptosis, which play key roles in cancer inhibition. Sudati et al.¹⁵ reported that *V. officinalis* ethanolic extract has an antioxidant effect against different toxic agents in rat brain homogenates. In addition, different studies showed the antioxidant effect of *V. officinalis*.¹⁶⁻¹⁸ Tian et al.¹⁹ reported that a *V. jatamansi* ingredient, valtrate, induced apoptosis in MDA-MB-231 and MCF-7 cells and inhibited cell migration. In another study, Han et al.²⁰ reported that valeric acid, one of the important ingredients of valerian root extract, inhibits liver cancer development via histone deacetylase inhibition.

Quan et al.²¹ isolated different iridoids from *V. jatamansi* roots, and two of these iridoids showed inhibitory effects on human glioma stem cells. Sen-Utsukarci et al.²² reported that *V. alliariifolia* ethanolic extracts obtained with different EMs (EM1 and EM2) have a high antioxidant capacity. Additionally, two different ethanolic extracts had different IC_{50} values (EM1 > 200

µg/mL and EM2 < 10 µg/mL). Bos et al.²³ showed the cytotoxic effects of different ingredients isolated from *V. officinalis* on human small-cell lung cancer cell and human colorectal cancer cell line (COLO 320) with MTT test. Valerenic acid and its derivatives, such as acetoxyvalerenic acid, hydroxyvalerenic acid, and methyl valerenate, which are obtained from *V. officinalis*, exhibited extremely low toxicity in both cell lines at the 100–200 µM concentration range.²³ In our study, the calculated IC_{50} values of *V. officinalis* methanolic extracts were 939.68 µg/mL for HepG2 cells and 1097.58 µg/mL for Caco2 cells. Differences in cytotoxicity results among studies of *Valeriana* species may depend on the different subtypes of the plant, plant section differences, EM differences, and study cell type.

In our study, the exposure of HepG2 and CaCo2 to the methanolic extract of *V. officinalis* in all exposure groups did not induce significant ROS production levels. Our results indicate that the methanolic extracts of *V. officinalis* may not induce cell death in cancer cell lines via oxidative stress induction. These results can be associated with commercial products obtained from different herbalists, which are less effective or ineffective in cancer cells.

CONCLUSION

In conclusion, the effects of *V. officinalis* extracts on cancer cells should be analyzed in detail with further studies that include different extraction protocols and with cultivated medical plants to define the anticancer effects of *V. officinalis*.

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