Original Article
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In-vitro Cytotoxicity and Oxidative Stress Evaluation of Valerian (*Valeriana officinalis*) Methanolic Extract in Hepg2 and Caco2 cells

Valeriana officinalis'in Methanol Ekstresinin HepG2 ve Caco2 hücrelerinde In-Vitro Sitotoksisite ve Oksidatif Stres Değerlendirmesi

Short title: Valeriana effects on cellular oxidative stress
Türkçe Kısa Başlık: Hücresel oksidatif stres üzerinde Valeriana'nın etkisi

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ABSTRACT
Introduction: Traditional treatment methods are becoming popular and commonly using in many societies and have become the first treatment for many people. Although some of these methods are useful, they may interact with drugs that a person is using for another illness and may cause various life-threatening risks. Valerian (Catweed) plant is used in traditional medicine applications due to its sedative effects for sleep regulating purposes. It has also been reported that valerian may exert anti-cancer effect in vitro.
Methods: In this study, cytotoxicty and oxidative stress effects of the extract of Valerian root were evaluated in Hepg2 and Caco2 cell lines. Cytotoxicity was evaluated via MTT test. Total-Reactive oxygen species (Total-ROS) analysis was performed via 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) assay in flow-cytometry.
Results: IC50 values were calculated as 936,6 µg/ml in Hepg2 and 1097,5 µg/ml in Caco2 cell lines. It has been observed that Valerian root extract was not induced oxidative stress in HepG2 and Caco2 cell lines.
Discussion and Conclusion: These results indicate that the valerian root extracts usage in cancer treatment as an alternative method could not effective and may cause a risk for public health, in the other hand due to not inducing oxidative stress it could be safe in tolerated concentrations which advised.

Keywords: *Valeriana officinalis*, HepG2, Caco2, Oxidative stress, MTT
ÖZET
Giriş-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. **Yöntem:** Bu çalışmada, Kediotu kökü ekstresinin sitotoksisite ve oksidatif stres etkileri Hepg2 ve Caco2 hücre hatlarında değerlendirilmiştir. Sitotoksisite değerlendirmesi MTT testi ile gerçekleştirildi. Total-reaktif oksijen bileşikleri (Total-ROS) analizi, hücre akış sitometrisinde 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) testi ile gerçekleştirildi
Bulgular: IC50 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 gözlenmiştir.
Tartışma ve 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

INTRODUCTION

The use of herbal products for treatment of several diseases has been widespread implementation from past to present. In developed countries, herbal remedies constructed as over-the counter drugs with on a tight leash. However, in developing countries herbal therapy methods and products using out of hand. Most of herbal drugs in the market have not fully evaluated from toxicological aspect which may cause several adverse effects of these products during the therapy in different body systems (1).
Valeriana genus belongs to the valerianaceae family that include approximately 300 species. *Valeriana officinalis* has been generally used as alternative traditional medicine for insomnina and depression therapy. This medical plant has sedative, anticonvulsant, hypnotic, and anxiolytic effects. Also it has been reported that this plant has treatment potential for gastrointestinal and urinary system problems. *V.officinalis* dependent on extraction methods include flavonoids, monoterpenes, sesquiterpenes, valepotriates, iridoids, alkaloids, acids like gamma-aminobutyric acid, glutamine and lignans which effects central nervous system; and also have antioxydant and vasorelaxant effects. Due to these effects *V.officinalis* is a popular herbal remedy choice to cure insomnia, headache, gastrointestinal system, cardiovascular system and urinary tract problems (2-5).
It has been speculated that Valeriana herbal remedy could be effective in cancer patients. Valerian herb ingredients anti-cancer effects have been took great concern by scientific area. However, in herbal therapy implementers have been trying to cure cancer patients with insufficient valerian products (6).
In this work, we have observed the cytotoxic and oxidative stress inducing effect, which are play important role in killing cancer cells, of methanolic extract *V.officinalis* in HepG2 and CaCo2 cell lines.

MATERIALS AND METHODS

Valeriana officinalis extraction

V. officinalis roots were purchased commercially from traditional herbal drug store in Istanbul. *V. officinalis* roots were pulverized in porcelain mortar. 75 ml methanol added on 15 g powderized *V.officinalis* root and incubated at room temperature with shaker for 24h. After incubation period extraction solution filtered with Whatmann no.1 filter and methanol was evaporated with fractional distillation (7).

Cell culture and 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 (ATCC, Virginia, USA), and cells were maintained as manufacturer's instructions. 1000 mg/ml extract was prepared by dissolving of V.officinalis root in 100% of dimethyl sulfoxide (DMSO), and stored in +4°C until the experiments. Before the cells treatments, extract diluted with DMSO that final DMSO concentration was 1%. Selected concentrations for cytotoxicity assay, root extract was dissolved in cell culture medium to prepare desired concentrations. Treatments were done at a concentration range for 24h to evaluate 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 ($1x10^4$ cells/100 µL cell culture medium/well). After overnight incubation cells were treated with *V.officinalis* extract at the concentration range of 200-400-600-800-1000 µM and control for 24h. In control group final DMSO concentration was arranged to 1%. Then 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was added into wells and then incubated for further 3h at 37 °C in the dark. Optical densities (ODs) were measured at 570 nm using a microplate reader (Biotek, Epoch, Vermont, USA) (8).

Total ROS assay with DC-FDA

Different pathological conditions were associated with reactive oxygen species (ROS) increase in the cell. Thus, detecting ROS level changes in basic in studies. Due to short half-life of ROS, effective detection methods are very important to observations. H_2DCFDA is a non-fluorescent dye and during ROS presence it returns green fluorescent with oxidation (9). In the present study, the ROS production was evaluated with H_2DCF-DA dye with by flow cytometer. The $5x10^5$ HepG2 and Caco2 cells in 2 ml of medium per well were seeded into 6-well plates and incubated overnight. The cells were treated with 100-200-100-600 µg/ml concentrations. These concentrations exerted cell viability higher than 70% depend on MTT assay for 24h. 1 % DMSO solution was used as the negative control for experiment. After 24h the cells were washed with PBS twice and incubated with 20 µM H_2DCF-DA at 37°C for 30 min. The cells were detached with trypsin-EDTA after incubation period and washed with PBS. Cells were re-suspended with 1% BSA in 150 µl PBS. The fluorescence intensity of 10^4 cells was measured with ACEA NovoCyte flow cytometer (San Diego, California, USA) and the results were expressed as 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 $\pm$ standard deviation. The statistical comparisons were analyzed using the One-way ANOVA analysis of variance followed by the 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 % cell inhibition of both Caco2 and HepG2 cell lines with *V.officinalis* methanolic extract exposure increased, concentration dependent for 24h (Figure 1 and Table 1). Inhibition concentration 50 (IC50) values were calculated via dependent to

graph slope formulations. IC50 values were 939,68 µg/ml for HepG2 cells and 1097,58 µg/ml for Caco2 cells.

Total-ROS Induction

Inhibition concentration 30 (IC30) values of *V.officinalis* methanolic extract on HepG2 and Caco2 cell lines were calculated for Total-ROS evaluation. IC30 values calculated as 600,12 µg/ml for HepG2 cells and 672,95 µg/ml for CaCo2 cells. 100-200-400 and 600 µg/ml concentrations were chosen for Total-ROS analysis for determine ROS production with flow-cytometer via H_2DCF-DA. There was no statistically significant difference between concentration groups for mean fluorescence index (MFI) for both cell lines. *V.Officinalis* methanolic extract did not cause increase of ROS production for 24h exposure (Figure 2).

DISCUSSION

Valerian is belonging to valerianaceae family that exist approximately 300 species only in western countries, and include several different phytochemicals that could have nervous system protection, diuretic, antispasmodic, anthelmintic, antioxidant, antimicrobial, anti-inflammatory, antirheumatic sedative, anticonvulsant and diaphoretic effects (6). Medicinal herbal plant valerian is important in traditional therapy on sleeping and anxiety disorders due to its effects on c-aminobutyric acid (GABA) A receptor (GABA(A)R) system (11). *Valeriana officinalis* products widely used for different type of diseases. In recent years, Valerian products usage in cancer cure increased by herbal implementers (2).

Several people die worldwide due to different cancer types and new therapeutic development studies always get interest by scientists. Oxidative stress induction plays important role in cancer cell death induction via chemotherapeutics. Oxidative stress has bivious effect on cancer cell induction and cancer cell death mechanisms. It could be inducing cancer cell proliferation via DNA damage or could be a therapeutic strategy to cure cancer via inducing cancer cell death through 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, Oliveria and Barreto (2009) reported that aqueous *V.officinalis* extract was exert protective role against apoptosis with rotenone exposure (2). Wang et al., (2017) showed *V.jatamansi* antioxidant effects on Human liver carcinoma (HepG2), human cervix carcinoma (HeLa), breast cancer (MDA-MB-231) and human umbilical vein endothelial (HUVECs) cells (12). Kakekashi et al., (2014) was reported that, *V. Sitchensis* adding in drinking water of male F344 rats, significantly supressed the 8-hydroxy-29-deoxyguanosine generation after hepatocarcinogenesis initiation by diethylnitrosamine with 50, 500 and 5000 ppm concentrations. Antioxydant catalase levels and apoptosis were increased and cell proliferation was significantly decreased. Apoptosis induction was associated with c-myc, Mafb, cyclin D1 expression supression and p21Waf1/Cip1, p53 and Bax expressions increase levels. These results indicate that V. Sitchensis administration could be associated with carcinogenesis inhibition (11). Zhu et al., (2019) were shown anticancer effects of *V. jatamansi* F3 fraction in vitro and in vivo. In MCF-10A cells F3 fraction administration was increased intracellular ROS production and induced apoptosis which were play key role in cancer inhibition (14). Sudati et al., 2009 reported that *V.officinalis* ethanolic extract have anti-oxidant effect in rat brain homogenates against different toxic agents (15). Also, there are different studies in the literature that shown anti-oxidant effect of *V.Officinalis* (16-18). Tian et al., (2020) was reported that, V.jatamansi ingrediaent Valtrate was induced apoptosis MDA-MB-231 and MCF-7 cells and inhibited cell migration. In another study, Han et al., (2020) reported that, valeric acid, one of the important ingredient of valerian root extract, inhibits liver cancer development via histone deacetylase inhibition (19,20).

Quan et al., (2020) isolated different iridoids from *V.jatamansi* root and two of these iridoids were shown inhibitory effects on human glioma stem cells (21). Şen-Utsukarçi (2019)

reported that, *V. alliariifolia* ethanolic extracts with different extraction methods (EM1 and EM2) have higher antioxidant capacity. Additionally, two different ethanolic extract have different IC50 values (EM1>200 µg/ml and EM2 < 10 µg/ml) (22). Bos et al., (1998) were shown the cytotoxicity different ingredients that isolated from of V.officinalis effects of on a human small-cell lung cancer cell (GLC_4) 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 that from *V. officinalis*, were shown very low toxicity with in both cell lines in 100 - 200 µM concentration range (23). In our study, V.officinalis methanolic extracts IC50 values were calculated as 939,68 µg/ml for HepG2 cells and 1097,58 µg/ml for Caco2 cells. Cytotoxicity results differences of Valeriana species between studies might be depends on different subtype of plant, plant section differences, extraction method differences and study cell type.

In our study we found that, methanolic extract of *V.officinalis* exposure on HepG2 and CaCo2 in all exposure groups did not induce ROS production levels. Our results could indicate that *V.officinalis'* methanolic extracts might not induce cell death in cancer cell lines via oxidative stress induction. These results could be associated with commercial products that obtained from different herbalists that results less effective or ineffective in cancer cells. In conclusion, *V.officinalis* extracts effects on cancer cells should analyze detailed with further studies that include different extraction protocols and with medical cultivated plants could be define anti-cancer effects of *V.officinalis*.

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Competing Interests: The authors declare that there are any non-financial competing interests.

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Table 1. MTT results of V.Officinalis extract exposure		
Concentration (µg/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

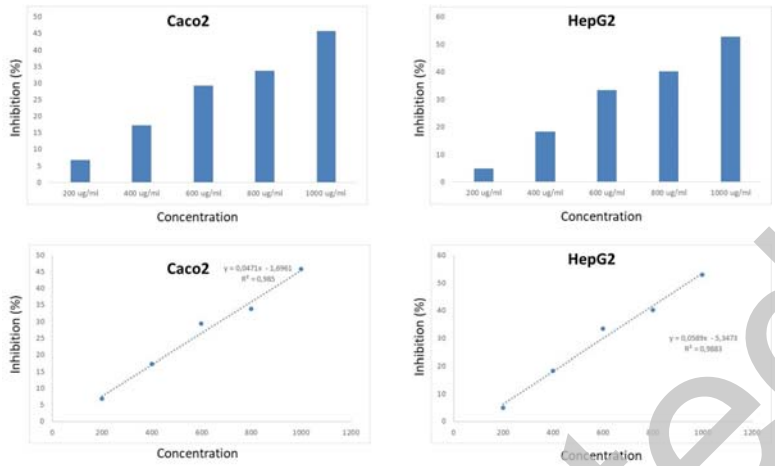

Figure 1. V.Officinalis methanolic extract cell viability inhibition (%) values on HepG2 and Caco2 cell lines increased concentration dependent. Graph slope formulations were shown on tha graphs.

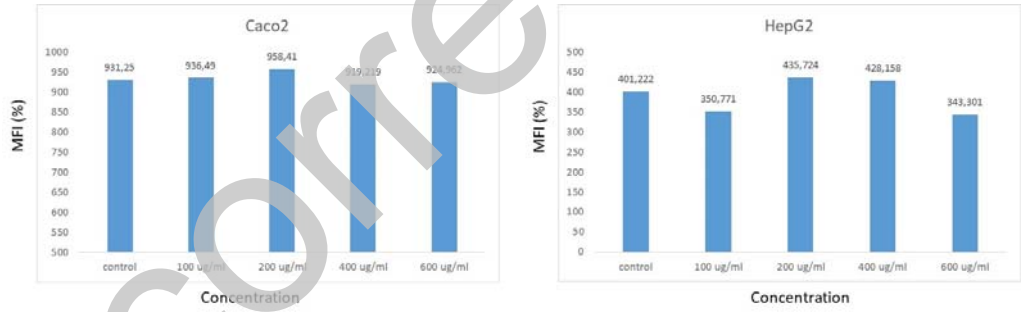

Figure 2. DC-FDA analysis results for HepG2 and Caco2 cell lines of 24h exposure of V.Officinalis methanolic extract. ROS production was not induced (p>0,05).