# CYTOTOXICITY EVALUATION of SOME PHENOLIC COMPOUNDS in V79 CELLS

Merve BACANLI[1,*], A. Ahmet BAŞARAN[2] and Nurşen BAŞARAN[1]

[1]Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Hacettepe University, 06100, Ankara, Turkey

[2]Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, 06100, Ankara, Turkey

***Corresponding author:** Merve BACANLI

Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

Phone Number: +90 (312) 305 21 78

e-mail address: merveg@hacettepe.edu.tr

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[2]Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, 06100, Ankara, Turkey

## SUMMARY

Phenolic compounds play an important role on the growth and reproduction of plants and also exhibit a wide range of pharmacological activities such as anti-allergic, anti-artherogenic, anti-inflammatory and anticancer. But the data about toxicity of many phenolics are lacking and limited. The aim of this study was to evaluate the cytotoxic effects of five different commonly used phenolic compounds (galangin, limonene, naringin, puerarin and ursolic acid) which are widely used because of their health protecting effects by neutral red uptake (NRU) assay in Chinese hamster fibroblast cell line (V79). It is found that all of the studied phenolics decreased the cell viability of V79 cells in a dose dependent manner. The IC50 values of galangin, limonene, naringin, puerarin and ursolic acid were found to be 104,36 μM, 10574 μM, 1976,4 μM, 51,71 μM and 92,94 μM, respectively.

***Key Words:*** Galangin, limonene, naringin, puerarin, ursolic acid, neutral red uptake assay

## BAZI FENOLİK BİLEŞİKLERİN SİTOTOKSİSİTESİNİN V79 HÜCRELERİNDE DEĞERLENDİRİLMESİ

## ÖZET

Fenolik bileşikler bitkilerin büyüme ve gelişmesinde önemli rol oynarlar ve ayrıca antiallerjik, antiarterojenik, antienflamatuvar ve antikanser etkiler gibi çok sayıda farmakolojik aktivite gösterirler. Ancak pek çok fenolik maddenin toksisitesine ilişkin very sınırlı ve az sayıdadır. Bu çalışmanın amacı, sağlığı koruyucu etkilerinden dolayı yaygın olarak kullanılan beş farklı fenolik bileşiğin (galangin, limonen, naringin, puerarin ve ursolik asit) Çin hamster fibroblast hücrelerinde (V79) sitotoksik etkilerinin nötral kırmızı alım (NKA) yöntemiyle değerlendirilmesidir. Çalışılan tüm fenolik bileşiklerin V79 hücrelerinin canlılığını doz artışıyla birlikte azalttığı bulunmuştur. Galangin, limonen, naringin, puerarin ve ursolik asit için IC50 değerleri sırasıyla 104,36 μM, 10574 μM, 1976,4 μM, 51,71 μM and 92,94 μM olarak bulunmuştur.

***Anahtar Kelimeler:*** Galangin, limonen, naringin, puerarin, ursolik asit, nötral kırmızı alım yöntemi

## INTRODUCTION

Phenolic compounds are secondary metabolites of the derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants [1]. These compounds play an important role onthe growth and reproduction in plants [1] and afford protection against ultraviolet radiation, pathogens, and herbivores [2]. Phenolic compounds also exhibit a wide range of pharmacological activities such as anti-allergic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, anticancer and cardioprotective [1]. Due to the cytotoxicity profile of many phenolic compounds, it is suggested that these chemicals can inhibit the survival of cancer cells. But the data about the cytotoxicity of these compounds in healthy cells are limited.

Galangin (3,5,7-trihydroxyflavone), belongs to a class of phenolic compounds known as flavonols [3]. It is present at high concentrations in propolis and in an Indian root, *Alpinia officinarum,* which is a common spice in Asia [4]. Galangin has been suggested to have biological activities including antimutagenic, antioxidant, anti-inflammatory, antiviral and anticarcinogenic [5, 6] .

Limonene (p-mentha-1,8-diene), is the major component of oils obtained from orange, grape fruit and lemon. This monocyclic monoterpene has shown chemopreventive and therapeutic activity against a wide variety of experimental tumors [7]. Limonene has variety of uses such as industrial degreaser, household cleaning agent, food and cosmetic flavoring and pesticide [8].

Naringin (4',5,7-trihydroxy flavanone 7-rhamnoglucoside) is a flavanone also present in grape fruit [9], which exhibits diverse biological and pharmacological properties including anti-inflammatory, antioxidant, anticarcinogenic and lipid-lowering activities [10].

Puerarin (daidzein-8-C-glucoside) is the major isoflavone derived from the root of *Pueraria lobata* (kudzu root) [11]. It is used in alternative medicine because of its estrogenic and antioxidant properties. In experimental models it is also suggested to be used in the prevention and treatment of cardiovascular diseases, diabetes, cancer and osteoporosis [12].

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenoid obtained from plants. It has long been used in Chinese medicine as anti-inflammatory, anti-arthritic, cytostatic and anti-proliferative, hepatoprotective agent[13].

The aim of this study was to evaluate the cytotoxic effects of these commonly used phenolic compounds (galangin, limonene, naringin, puerarin and ursolic acid) by neutral red uptake (NRU) assay in Chinese hamster fibroblast cell line (V79).

## MATERIALS and METHODS

### *Chemicals*

The chemicals used in the experiments were purchased from the following suppliers: fetal calf serum (FCS), trypsin-EDTA, penicillin-streptomycin, from Biological Industries (Kibbutz Beit-Haemek, Israel), minimum essential medium (MEM), dimethyl sulfoxide (DMSO), Triton X-100, phosphate buffered saline (PBS), ethanol, neutral red (NR), galangin, naringin, ursolic acid from Sigma (St Louis, USA), limonene and puerarin from Fluka (St. Gallen, Switzerland).

### *Cell Culture*

Cells were seeded in 75 $cm^2$ flasks in 20 ml MEM supplemented with 10% FCS and 1% penicillin-streptomycin and then grown for 1 day in an incubator at 37°C in a humidified atmosphere supplemented with 5% $CO_2$.

***Determination of Cytotoxicity by NRU Assay***

The cytotoxicity of phenolic compounds was performed in V79 cell line by NRU assay following the protocols described by Virgilio et al. (2004) [14] and Saquib et al. (2012) [15].

Following disaggregation of cells with trypsin/EDTA and resuspension of cells in the medium, a total of $10^5$ cells/well were plated in 96 well tissue-culture plates. After 24 h incubation, the different concentrations of galangin, limonene, naringin, puerarin and ursolic acid in medium were added. The cells were incubated for 18 h (1.5 cell cycle) at 37°C in 5% $CO_2$, then the medium was aspirated. The cells were washed twice with PBS and incubated for an additional 3 h in the medium supplemented with NR (50 µg/ml). After the medium was discarded, the cells were rinsed five times with warm PBS (37°C) to remove the nonincorporated excess dye and 200 µl of "fixation solution" (50% ethanol, 1% acetic acid, and 49% distilled water) was added to each well to fix the cells and bring NR into solution. The plates were shaken for 20 min, and the absorbance of the solution in each well was measured in a microplate reader at 540 nm and compared with the wells containing untreated cells. Results were expressed as the mean percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as the percent of control (assuming data obtained from the absence of phenolic compounds as 100 %). IC50 values represent the concentrations that reduced the mean absorbance of 50% of those in the untreated cells.

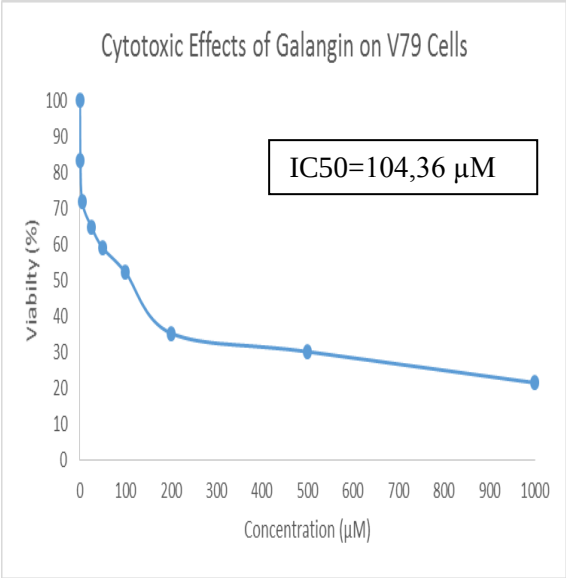
## RESULTS

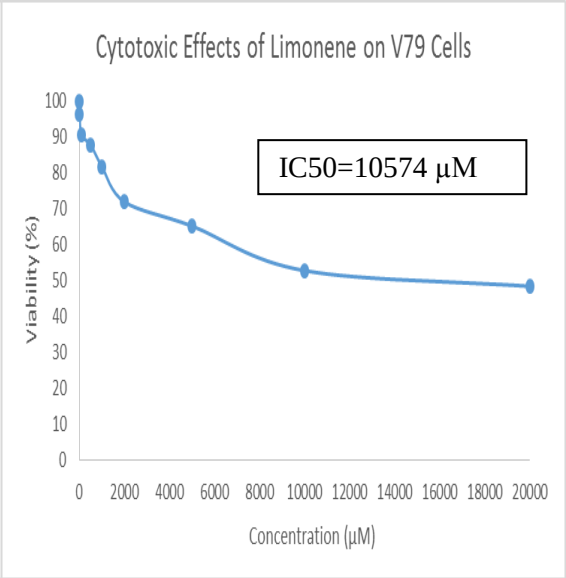
A concentration dependent decrease was seen in the survival of cells exposed to galangin, limonene, naringin, puerarin and ursolic acid (Figure 1). IC50 values of galangin, limonene, naringin, puerarin and ursolic acid in V79 cells were found to be 104,36 µM, 10574 µM, 1976,4 µM, 51,71 µM and 92,94 µM respectively.

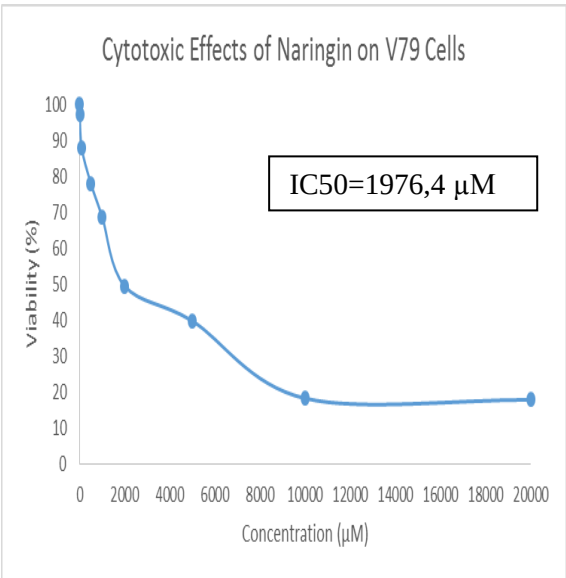
## DISCUSSION

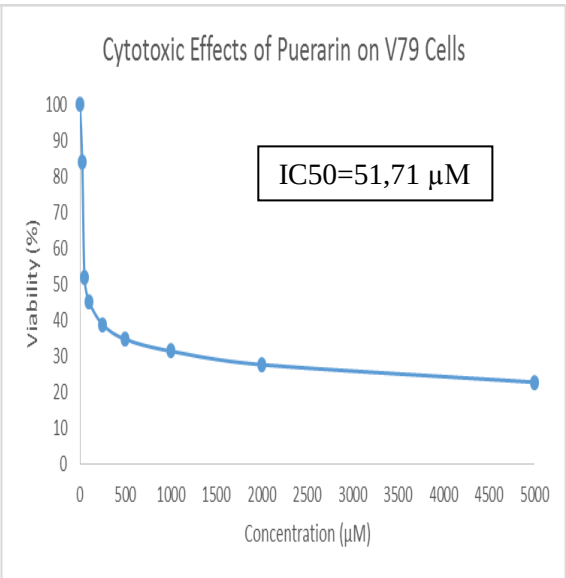
Several studies have showed that diets rich in fruits and vegetables can be associated with a markedly decreased risk of chronic diseases. This has been attributed to high levels of antioxidant contents of these foods [1]. Natural products are widely used as dietary supplements because of their potential antioxidant properties. On the other hand, it is suggested that at low doses phenolic compounds have antioxidant properties, but at high doses they can exert pro-oxidant effects[16].

The word "cytotoxicity" has a broad and sometimes vague meaning [17]. With regard to in vitro cell culture systems, a test substance is considered to be cytotoxic if it interferes with attachment of cells, significantly alters morphology, adversely affects the rate of cell growth, or causes cells to die [18].There has been much attention devoted to cytotoxicity studies as a first step in evaluating the toxicity of test substances. This is especially valid in connection with screening biological activity of plant extracts and active compounds isolated from plants[19].


Cytotoxic Effects of Galangin on V79 Cells
IC50=104,36 µM
Viability (%)
Concentration (µM)


Cytotoxic Effects of Limonene on V79 Cells
IC50=10574 µM
Viability (%)
Concentration (µM)


Cytotoxic Effects of Naringin on V79 Cells
IC50=1976,4 µM
Viability (%)
Concentration (µM)


Cytotoxic Effects of Puerarin on V79 Cells
IC50=51,71 µM
Viability (%)
Concentration (µM)

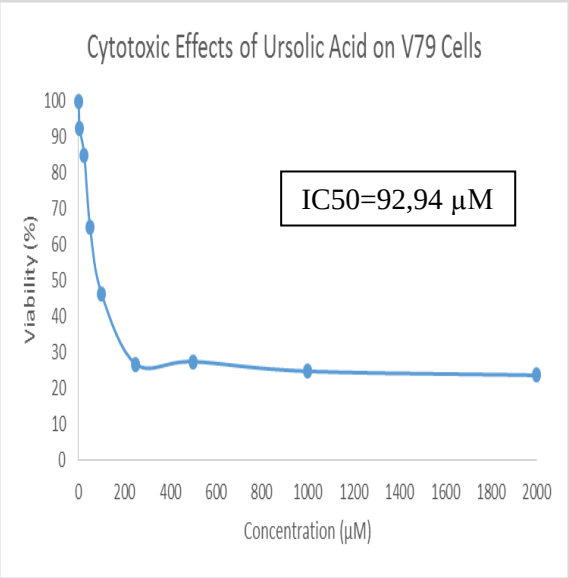



Figure 1. CytotoxicEffects of Galangin, Limonene, Naringin, PuerarinandUrsolicAcid in V79 Cells

V79 cells, a well-defined cell line, are widely used in toxicity studies [20]. These cells are available for cytotoxicity studies. Because they do not have p450 enzyme system and they can keep basal cell functions in normal cell culture conditions [21].

Galangin, a member of the flavonol class of flavonoid, used in many countries for the management of various diseases, including airway affections, viral infections [5], and found to have antioxidant [22], anticancer [23] and antibacterial [24] activities. The data about the cytotoxicity of galangin is limited. In a previous study, gastric cancer (SNU-484) cells were incubated with galangin for 24 hours then cell viability was evaluated by MTT assay. The cell viability was decreased with the increasing concentrations of galangin and the IC50 value of galangin was found to be 100 µM in these cell lines[25]. The cytotoxic effects of galangin were increased depending on the doses in liver cancer (HepG2 and PCL/RPF/5) cells and cellcycles of three celllineswereblockedbygalangin[26]. In our study, we found that cell viability of V79 cells was decreased with the increasing doses of galangin and IC50 value of galangin was found to be 104,36 µM. It is found that galangin has no toxic effects under 100 µM concentration in different cells.

Limonene, a monocyclic monoterpene, is the major constituent of*Citrus* fruits is widely used as a flavor and fragrance additive because of its pleasant citrus fragrance. Limonene is listed in the Code of Federal Regulation as generally recognized as safe (GRAS) for flavoring agent [27]. This monocyclic monoterpene has shown to be have chemopreventive and therapeutic activity against a wide variety of diseases [7]. The cytotoxic effects of *Citrus sinensis* essential oil was evaluated on human adenocarcinoma (SW480 and HT-29) cells. The cell viability was decreased at 6.25-500 ppm concentration with 24, 48 and 72 h incubations [28]. In a different cytotoxicity study with limonene, the cytotoxic effects were increased in a dose dependent manner in human lung adenocarcinoma (A549) and HepG2 cell lines and IC50 values were found to be 586 µM and 889 µM, respectively. In our study, in healthy V79 cells the IC50 value oflimonene was found to be 10574 µM.

Studies about naringin have focused on its health preventive and antioxidant effects [29]. Our study is the first study about the cytotoxicity of naringin. In our study, IC50 value of naringin was found to be 1976,4 µM. It is found that naringin has no toxic effects under 2000 µM concentration in V79 cell line.

Puerarin has also been suggested to have health promoting effects against

cardiovascular and gynecological diseases, osteoporosis and diabetic nephropathy [30]. There is limited data about the cytotoxicity of puerarin. In a study, it is claimed that puerarin has blocked proliferation of HT-29 cells in a dose and time dependent manner [31]. In our study, we found that cell viability of V79 cells was decreased in a dose dependent manner and IC50 value of puerarin was found to be 51,71 μM.

Ursolic acid is naturally found in several medicinal herbs including rosemary, thyme, oregano and lavender. It possesses several biological activities such as anti-inflammatory, antiangiogenic, antiviral, antioxidant, antihypertensive, antihyperlipidemic and antihyperglycemic [32, 33]. In a study with colon cancer (CaCo-2) cells, ursolic acid decreased the cell viability at the higher concentrations in MTT assay [34]. In our study, we found that cell viability of V79 cells was decreased with the increasing doses of ursolic acid and IC50 value of ursolic acid was found to be 92,94 μM.

In conclusion, in this study the cytotoxic effects of galangin, limonene, naringin, puerarin and ursolic acid were examined. All of the studied phenolics were decreased the cell viability of V79 cells with increasing dose in NRU assay. Further investigation such as using more cell lines and different reliable cytotoxicity assays and incubations with various concentrations at many time points should be performed to corfirm beneficial and toxic effects of phenolics.

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