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Assesment of Cytotoxic and Genotoxic Properties of Phenolic Compounds and Hydrolysable Tannins from Geranium psilostemon Ledeb.

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Abstract

Geranium species are medicinal food plants used as tonic, diuretic, antidiabetic, antidiarrheal, and antihemorrhoidal in traditional medicine. *Geranium psilostemon* Ledeb. (Geraniaceae), which has a rich phenolic content, grows widely in Turkey. The aim of this study is to evaluate cytotoxicity and genotoxicity of the compounds isolated from *G. psilostemon*. Cytotoxic effects of the compounds were determined by neutral red uptake (NRU) assay. COMET assay was used for assesing genotoxic effects of the compounds. IC_{50} values of the compounds were calculated in different cell lines to evaluate cytotoxicity. 1,3,6-tri-*O*-galloyl-*β*-glucopyranose showed the most cytotoxic effect on L1210 and V79 cell lines and IC_{50} values of the compound were 3.7 and 13 µg/ml, respectively. Besides, in HeLa cell line, 1,3,6-tri-*O*-galloyl-*β*-glucopyranose and gallic acid had the lowest IC_{50}, 18 and 15 µg/ml, respectively. All the compounds exhibited significant cytotoxic effects at all concentrations. Besides, they also showed genotoxic activity at 50 µg/ml. The tested compounds isolated from *G. psilestemon*, a medicinal food plant, have cytotoxic and genotoxic potential. Therefore, it should be considered regarding these biological acitivities. Further studies are necessary to determine the optimal concentrations of the compounds for evaluating their anticancer and other biological activities.

Keywords: phenolic compound, hydrolysable tannin, cytotoxicity, genotoxicity

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Özet

Geranium psilostemon Ledeb. bitkisinden elde edilen fenolik bileşikler ve hidrolize olabilen tanenlerin sitotoksik ve genotoksik özelliklerinin değerlendirilmesi

Geranium türleri, geleneksel tıpta tonik, diüretik, antidiyabetik, antidiyareal ve antihemoroidal olarak kullanılan tıbbi gıda bitkileridir. *Geranium psilostemon* Ledeb. (Geraniaceae), fenolik bileşiklerce zengin, Türkiye'de yaygın olarak yetişen bir türdür. Bu çalışmanın amacı, *G. psilostemon*'dan elde edilen bileşiklerin sitotoksisitesinin ve genotoksisitesinin değerlendirilmesidir. Bileşiklerin sitotoksisitesi neutral red uptake (NRU) yöntemi ile belirlenmiştir. Genotoksik etkilerinin değerlendirilmesinde COMET kullanılmıştır. Bileşiklerin IC_{50} değerleri farklı hücre kültürlerinde sitotoksisitenin değerlendirilmesi için kullanılmıştır. 1,3,6-tri-*O*-galloil-*β*-glukopiranoz L1210 ve V79 hücrelerinde en çok sitotoksik etkiyi göstermiştir ve IC_{50} değerleri sırasıyla 3.7 ve 13 µg/ml'dır. Bunun yanısıra, HeLa hücrelerinde, 1,3,6-tri-*O*-galloil-*β*-glukopiranoz ve gallik acit en düşük IC_{50}, (sırasıyla 18 and 15 µg/ml) değerine sahiptir. Tüm bileşikler kullanıldıkları bütün konsantrasyonlarda sitotoksik etki göstermiştir. Ayrıca, 50 µg/ml konsantrasyonda genotoksik etkileri de gösterilmiştir. *G. psilestemon'*dan izole edilen test bileşikleri, tıbbi gıda bitkileri olup sitotoksik ve genotoksik potansiyelleri bulunmaktadır. Dolayısıyla, bu biyolojik aktivitelerinin de değerlendirilmesi gerekir. Antikanser ve diğer biyolojik aktivitelerinin değerlendirilebilmesinde optimum konsntrasyonların belirlenmesi için ileri çalışmalara ihtiyaç vardır.

Anahtar kelimeler: fenolik bileşik, hidrolize olabilen tane, sitotoksisite, genotoksisite

1. Introduction

Natural compounds have been widely used for prevention of various diseases for centuries. For the last decade, isolated bioactive products have been important sources for the development of new drugs. Diverse and complex chemical structures and different activites of natural products attract the attention of scientists. Turkey has a rich flora, which is a good alternative for natural product researches [1].

There are more than 400 species of *Geranium* plants, which widely grow all over the world and are used for their antidiabetic, hemostatic, antihemorrhoidal, and antidiarrheic effects. Therefore, it has been reported that these species are used for the treatment of different pathological conditions such as cancer, fever, tonsillitis, cough, urticaria, dysentery, pain, and gastrointestinal illnesses [2-6]. *Geranium* species are consumed as salad in Turkey and Russia [5-7]. *Geranium* species have various active compounds such as flavonoids and tannins [4,9]. Genus *Geranium* is represented by 35 species in Turkey. Among these, *Geranium psilostemon* Ledeb. is a perennial plant which grows naturally only in Eastern Black Sea Region of Turkey, Armenia, Azerbaijan, and southwest part of Russia [1,10]. In addition, antioxidant, cytotoxic, antiinflam-

matory, antiviral, and antidiabetic activity studies were performed on these species and it was determined that some of the species were very potent [4,9,11-15].

Several flavonoids, tannins, and other phenolic compounds have been isolated from *Geranium* species so far [4, 9, 16]. The phenolic compounds play conflicting and complex roles as radical scavengers, antioxidants, and prooxidants [17-19]. Besides, cytotoxic activity of hydrolysable tannins on several cancer cells have been shown in different studies [20, 21].

Phenolic compounds are commonly found in fruits, vegetables, chocolate, and beverages such as tea, coffee, and wine and obtained from regular diet [22]. Dietary phenolic compounds are classified as phenolic acids, flavonoids, lignans, stilbenes, coumarins, and tannins [23, 24]. Tannins are catagorized into condensed tannins and hydrolysable tannins [25].

In our previous studies showed that the compounds obtained from *G. psilostemon* have a very high antioxidant potential [16]. According to the literature, it has been shown that the phenolic compounds have cytotoxic effects in different cell lines [20, 21]. In this study, the assessment of genotoxic and cytotoxic potential of simple phenolic compounds (gallic acid and methyl gallate) and hydrolysable tannins (pusilagin, 1,3,6-tri-O-galloyl-β-glucopyranose, 1,2,3,4,6-penta-O-galloyl-β-glucopyranose) (**Fig. 1**) obtained from *G. psilostemon* were evaluated.

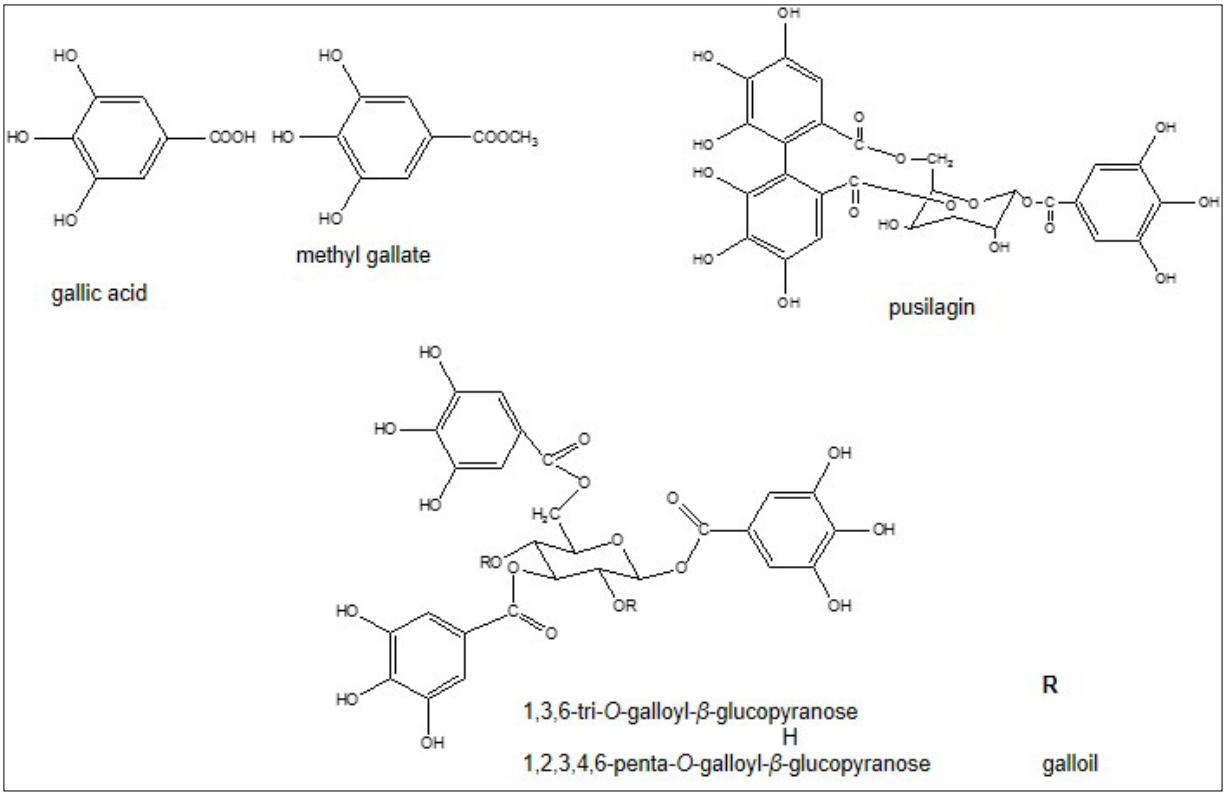

Figure 1. The chemical structures of tested compounds, 1,3,6-tri-*O*-galloyl*β*-glucopyranose **(1)** pusilagin **(2)**; methyl gallate **(3)**; 1,2,3,4,6-penta-*O*-galloyl-*β*-glucopyranose **(4)**; gallic acid **(5)**

2. Materials and Methods

2.1. Cell Culture

V79 Chinese hamster lung fibroblast cells used in this study were obtained from (DSMZ Braunschweig, Germany). L1210, mouse lymphocytic leukemia cells and HeLa, human epitheloid cervix carcinoma cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were subcultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with penicillin/streptomycin and 10% fetal calf serum (FCS) from Invitrogen (Karlsruhe, Germany) as described below.

2.2. Plant Material

The plant was collected in August 2006 from Trabzon, Turkey. A voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 06003). The plant material was identified by Didem Sohretoglu.

2.3. Chemicals

Chemicals were purchased from different providers: hydrogen peroxide (H_2O_2), dimethylsulfoxide (DMSO), trypsin–EDTA, ethanol (HPLC grade), potassium peroxodisulphate, phosphate buffered saline (PBS) tablets, triton X-100, ethidium bromide (EtBr), 5-isopropyl-2-methylphenol (98%), and 2′,7′dichlorodihydrofluorescein diacetate (DCHF-DA), agarose MEEO from Carl Roth; sodium chloride (NaCl) and sodium hydroxide (NaOH) from Merck Chemicals; normal melting agarose (NMA) and low melting agarose (LMA) from Boehringer Mannheim; ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA), Na-lauroylsarcosinate and Tris from ICN.

2.4. Neutral Red Uptake (NRU) Assay

The neutral red accumulation assay was modified from Papis et al., 2011 [26]. In order to determine the effect of the compounds on cell viability, V79 cells were seeded in 96-well microtiter plates at $1x10^4$ cells/well. The cells were attached after 24 h and the exposure to the compounds was started at different concentrations (5 µg/ml, 10 µg/ml, 50 µg/ml, 500 µg/ml) for 48 h. After exposure period, 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 (pH 7.4) to remove the nonincorporated excess dye and 200 µl of 'destain solution' (50% ethanol, 1% acetic acid, and 49% distilled water) was added to each well to fix the cells and bring the 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 wells containing untreated cells.

2.5. The 50% Inhibitory Concentration (IC_{50}) Cytostatic Activity Assay

The cytostatic activity of the compounds was examined in different cell lines. For this purpose, adherent cells (Hela and V79) were seeded in 48-well microtiter plates at $1x10^4$ cells/well. After 24 h, the cells were exposed to different concentrations of the compounds and allowed to proliferate for 48 h. Then the cells were trypsinized and counted using a Coulter counter. Suspension cells (L1210) were seeded in 96-well microtiter plates at $6x10^4$ cells/well in the presence of different concentrations of the compounds. The cells were allowed to proliferate for 48 h and counted with a Coulter counter [27]. IC_{50} values were calculated as the compound concentration required to reduce cell proliferation by 50%.

2.6. Single-cell Gel Electrophoresis (COMET) Assay

The basic alkaline COMET assay which is also known as Single Cell Gel Electrophoresis assay was used [28, 29]. The assay was performed on V79 cells. The principle of the method is based on migration of DNA in an agarose gel under electrophoretic conditions. For the treatment, 1×10^6 cells were seeded in six well plates and incubated in DMEM medium with 10% FCS at 37°C in a 5% CO_2 atmosphere. 50 µg/ml concentrations of the compounds were used for the experiment. A negative control (1% DMSO) and a positive control (50 µM H_2O_2) were also included in the experiments. For visualisation of DNA damage, slides were examined under fluorescence microscope. Measurements of tail length, tail intensity, and tail moment of DNA were made for 100 randomly selected cells per slide by a computer-based image analysis system called 'Comet Assay III' Perceptive Instruments. The mean values of these parameters were calculated and used for the evaluation of DNA damage.

2.7. Statistical Analysis

The results obtained *in vitro* were statistically processed using Microsoft Excel program. Student's *t*-test was applied and $p < 0.05$ was accepted for statistical significance.

3. Results and Discussion

3.1. Neutral Red Uptake (NRU) Assay

Cytotoxic effects of the compounds on cell viability were determined for different concentrations (5 µg/ml, 10 µg/ml, 50 µg/ml, 500 µg/ml) by neutral red assay (**Figure 2**). They showed comparable cytotoxic activity to negative control at all tested concentrations. All the tested compounds showed cytotoxic effect at 500 µg/ml significantly different from other concentrations.

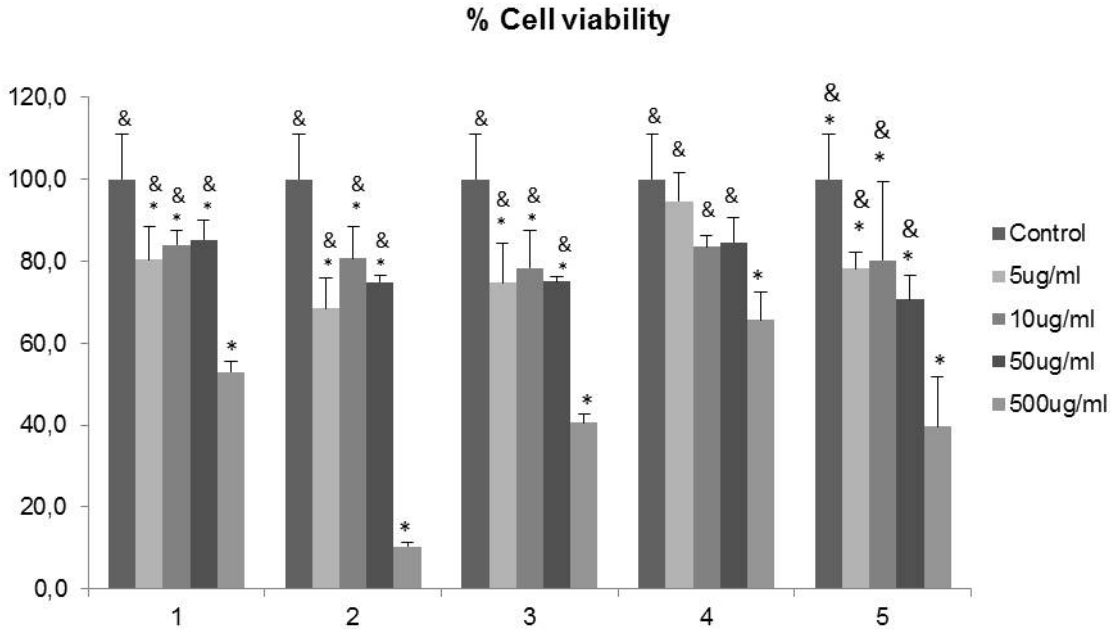

Figure 2. Effects of the compounds on the cell viability of V79 cells by neutral red **assay** at different concentrations (5 µg/ml, 10 µg/ml, 50 µg/ml, 500 µg/ml)**.** 1,3,6-tri-*O*-galloyl*β*-glucopyranose **(1)** pusilagin **(2)**; methyl gallate **(3)**; 1,2,3,4,6-penta-*O*-galloyl-*β*-glucopyranose **(4)**; gallic acid **(5)** *: significantly different from control $p<0.05$, &: significantly different from 500 µg/ml for the same compound. Results are expressed as **%** ±SD values of three observations.

3.2. The 50% Inhibitory Concentrations (IC_{50})

The cytotoxic evaluation of the compounds were made by determining IC_{50} levels in different cell lines. The results are shown in **Table 1**. Minimum and maximum IC_{50} values of the compounds were 3.7 -8.1 µg/ml for L1210 cells, 13-15 µg/ml for V79 cells and 18-15 µg/ml for HeLa cells, respectively.

Table 1. Cytostatic activity of the compounds as represented by the IC_{50} value in different cell lines. Data represent the mean (±S.D.) of at least two independent experiments

	IC_{50}* *(µg/ml)*		
Compound	***L1210***	***V79***	***HeLa***
1	3.7 ± 0.1	13 ± 3	18 ± 2
2	13 ± 1	21 ± 1	20 ± 1
3	14 ± 0	21 ± 0	20 ± 0
4	18 ± 2	44 ± 3	41 ± 25
5	8.1 ± 2.7	15 ± 3	15 ± 4

*50% inhibitory concentration.

3.2. Single-cell Gel Electrophoresis (COMET) Assay

In order to evaluate the genotoxic potential of the compounds, COMET assay was performed. Genotoxic activity of the compounds were evaluated by using tail moment, tail intensity, and tail length parameters. Results are given in Table 2.

Table 2. Genotoxic evaluation of the compounds by COMET assay. 1,3,6-tri-*O*-galloyl*β*-glucopyranose **(1)** pusilagin **(2)**; methyl gallate **(3)**; 1,2,3,4,6-penta-*O*-galloyl-*β*-glucopyranose **(4)**; gallic acid **(5).** Data represent the mean (%±S.D.) of two independent experiments

	1	2	3	4	5
Tail Length	36.06±7.90	39.69±12.74	45.06±19.49	51.04±23.20	39.63±16.9
Tail Intensity	14.86±23.20	32.47±27.10	18.50±23.47	36.79±28.19	30.16±30.16
Tail Moment	3.86±6.81	7.43±6.68	4.86±6.50	10.53±11.59	7.02±8.13

4. Discussion

The indicated activities of *Geranium* extracts are partially associated with the presence of various polyphenolic compounds which act as antioxidants by scavenging free radicals [30, 31]. However, apart from their beneficial properties, polyphenols may be toxic in mammalian cells besides their other modes of action. Cytotoxicity mechanisms of polyphenols are associated with the formation of their oxidation products [31, 32]. Phenolic compounds are considered as potential chemoprotective agents because of their biological activities in cells. Therefore, there are different hypotheses to explain their antitumoral activities including cytotoxic and antiproliferative effects. They also impact on cell differentiation and angiogenesis processes [22, 33].

Gallic acid and its esters, such as E-310 (propyl gallate), E-311 (octyl gallate), are used as antioxidant additives in both food and pharmaceutical industry. Besides, the cytotoxic effects of gallic acid and methyl gallate are also well studied. Cytotoxic activity of gallic acid **(5)** has

been reported in a variety of cancer cells, such as leukemia, skin, prostate, lung, stomach, colon, breast, cervix, and esophagus [33-41]. Cytotoxicity of methyl gallate **(3)** has been also shown in skin, cervix, and leukemia cancer lines [41]. Moreover, it has been reported in various test models that gallic acid **(5)** decreases or inhibits cancer cell migration and invasion [44].

In vitro inhibition of growth and invasiveness of breast cancer, leukemia, melanoma, colon, and liver cancer cells by 1,2,3,4,6-penta-*O*-galloyl-*β*-glucopyranose (**4**) has also been indicated in literature [39,40]. There are *in vivo* preclinical studies in which inhibition of prostate cancer, lung cancer, and sarcoma cells by the same compound was demonstrated as well [40,41].

There is not sufficient data regarding genotoxicity of the tested compounds in the literature. According to the experiments, the compounds have genotoxic effects compared to the negative control. We observed that compound 4 caused more DNA damage than the other compounds. On the other hand, the results were parallel with each other for all the parameters. Labieniech and Gabryela also showed gallic acid's genotoxic effect on Chinese hamster cells (B14), which is consistent with our data [41].

The data presented in this study show that the compounds which originated from *G. psilestemon* have cytotoxic and genotoxic potential. Therefore, consumed as food, this plant should be considered regarding these biological acitivities. Phenolic compounds at higher concentrations can cause inhibition of cell proliferation [14,18]. Tannins, a group of polyphenolic compounds widely distributed in plants, are often encountered in our daily diet, being present in foods, beverages, and medicinal plants. Several epidemiological studies have indicated that tannins may exert a protective effect against cancer. They have highly reactive phenolic groups in their structure [20,21]. Thus, with its rich phenolic content, this plant could also be benefited as an anticancer due to its cytotoxic effect, however one should keep in mind that it might have genotoxic potential.

The use of plant extracts is increasingly becoming widespread. Application of cytotoxicity and genotoxicity tests to these compounds could help to identify their activities and increase their safety. Therefore, further studies are necessary to determine the activities and optimal concentrations of the compounds from *Geranium psilostemon*.

On the basis of our data, high amounts of this plant and also others which contain the tested compounds must be consumed carefully in traditional medicine.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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