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Original article

Assessment of Cytotoxic Properties of Sinapic Acid *In Vitro*

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Phenolic compounds, a group of plant metabolites, are of considerable interest because of their antioxidant properties. Sinapic acid, a phenolic compound, is widely distributed in various fruits and vegetables. It is suggested to show some pharmacological effects such as antioxidant, antimicrobial, and anticancer. However, there is not enough data about the cytotoxicity of sinapic acid; the available data are limited. This study was aimed to assess the cytotoxic profiles of sinapic acid in a wide range of concentrations for 18 h exposure in two different cell lines, Chinese hamster lung fibroblasts (V79) and human cervical carcinoma (HeLa) cells using Neutral Red Uptake assay. The concentrations up to 500 µM and 2000 µM had no significant effect on V79 and HeLa cells, respectively, but the cell viabilities decreased below 50 % at concentrations higher than 1000 µM and 5000 µM for V79 and HeLa cells, respectively. IC_{50} values were found to be 1860 µM and 7248 µM in V79 and HeLa cells, respectively. This study has shown that sinapic acid have no cytotoxic effects in two different cell lines except at very high concentrations.

Key words: Sinapic acid, Cytotoxicity, Phenolic compounds, Neutral red uptake assay, Chinese hamster lung fibroblasts cells, Human cervical carcinoma cells

Sinapik Asidin Sitotoksik Özelliklerinin *İn Vitro* İncelenmesi

Bitki metabolitlerinin bir grubu olan fenolik bileşikler, antioksidan özellikleri nedeniyle oldukça fazla ilgi görmektedir. Sinapik asit fenolik bir bileşik olup çeşitli meyve ve sebzelerde yaygın bulunur. Antioksidan, antimikrobiyal, antikanser dahil pek çok farmakolojik etkiler gösterebildiği belirtilmektedir. Ancak sinapik asitin sitotoksisitesi hakkında yeterli veri bulunmamaktadır, mevcut veriler sınırlıdır. Bu çalışmanın amacı iki farklı hücre hattında; Çin hamster akciğer fibroblast (V79) ve insan serviks kanser (HeLa) hücreleri, sinapik asitin geniş bir konsantrasyon aralığında 18 saat maruziyetinde sitotoksik özelliklerini nötral kırmızı alım yöntemi ile değerlendirmektir. Sinapik asitin 500 µM ve 2000 µM'a kadar olan konsantrasyonları, sırasıyla V79 ve HeLa hücrelerinde, anlamlı bir etki göstermemiştir, ancak 1000 µM ve 5000 µM üzeri konsantrasyonlarında, sırasıyla V79 ve HeLa hücrelerinde hücre canlılıkları %50'nin altındadır. IC_{50} değerleri sırasıyla V79 ve HeLa hücreleri için 1860 µM ve 7248 µM olarak bulunmuştur. Bu çalışma sinapik asitin iki farklı hücre hattında genel olarak çok yüksek konsantrasyon haricinde sitotoksik etkilere sahip olmadığını göstermiştir.

Anahtar kelimeler: Sinapik asit, Sitotoksisite, Fenolik bileşikler, Neutral kırmızı alım yöntemi, Çin hamster akciğer fibroblast hücreleri, İnsan serviks kanser hücreleri

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INTRODUCTION

Phenolic compounds are a group of key plant metabolites found abundantly in fruits and vegetables. Because of their antioxidant properties, they exhibit a wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (1-3). The antioxidant activity of phenolic compounds depends on

the structure, in particular the number and the positions of the hydroxyl groups and the nature of substitutions on the aromatic rings (4). Phenolic compounds are present as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters and methyl esters (5,6).

Hydroxycinnamic acids (HCAs) are one of the major classes of phenolic compounds (7). Among the most common and well-known HCAs are cinnamic acid, o-coumaric acid, m-coumaric acid, p-coumaric acid, caffeic acid, ferulic acid, and sinapic acid (8).

Sinapic acid ($C_{11}H_{12}O_5$) (3,5-dimethoxy-4-hydroxycinnamic acid) is widely distributed in various fruits, vegetables, cereal grains, oilseed crops, some spices and medicinal plants (9). The amount of sinapic acid in plants has a wide range such as with 0.07 to 0.14 µg/g for cereal grains, 72.1 µg/g for lemon (*Citrus limon* L.), 450 µg/g for strawberries (*Fragaria ananassa* L.) and 1210 µg/g for dill (*Anethum graveolens* L.) (10-13). The maximum level of sinapic acid in plasma after consumption of the meal has been around 40 nM, indicating the absorption occurs mostly through the small intestine (14). The concentration of sinapic acid in human plasma after consumption of cranberry juice was found to be 1.5 µg/mL (15).

Sinapic acid shows many pharmacological activities almost in all systems. Several *in vitro* and *in vivo* studies have been conducted to determine the pharmacological properties such as antioxidant, antimicrobial, anti-inflammatory, analgesic, and anticancer of sinapic acid and to elucidate mechanism of action of this agent (9). Sinapic acid is assumed to be therapeutically beneficial and generally not toxic. However, up to now, there is not enough data about the cytotoxicity of sinapic acid; the available data are very limited.

The aim of this study was to determine the cytotoxic profile of sinapic acid in two different cell lines, Chinese hamster lung fibroblasts (V79) and human cervical carcinoma (HeLa) cells.

EXPERIMENTAL

Chemicals

The chemicals used in the experiments were purchased from the following suppliers: acetic acid, dimethyl sulfoxide (DMSO), Dublecco's modified Eagle's medium (DMEM), ethanol, methanol, ethylenediamine tetra acetic acid disodium salt dihydrate (Na_2-EDTA), trypan blue, 3-amino-m-dimethylamino-2-methyl phenazine hydrochloride (neutral red), penicillin-streptomycin, sinapic acid, sodium chloride (NaCl), sodium hydroxide (NaOH) from Sigma (St. Louis, MO, USA), fetal bovine serum (FBS), trypsin–EDTA, RPMI 1640 medium, L-glutamin, Dulbecco's phosphate buffered saline (PBS) from Biological Industries (Kibbutz Beit-Haemek, Israel); millipore filters from Millipore (Billerica, MA, USA), all other plastic materials from Cornings (Corning Inc., NY, USA).

Cell culture

V79 (Chinese hamster lung fibroblasts cell line) and HeLa (human cervical adenocarcinom cell line) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). V79 cells were grown in RPMI1640 medium supplemented with 10% heat-inactivated FBS, and 1% penicillin- streptomycin solution (10000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl), and 2mM L-glutamin at 37°C in a humidified atmosphere of 5% CO_2. HeLa cells were grown in DMEM supplemented with 10% heat-inactivated FBS, and 1% penicillin-streptomycin solution (10000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) at 37°C in a humidified atmosphere of 5% CO_2. The cells were subcultured in 75 cm^2 cell culture flasks. The culture medium was changed every 3 to 4 days. The passage numbers used in our study for both cell lines were between 6 and 10.

Determination of cytotoxicity

After growing for 2 weeks, cells were plated at 1×10^4 cells/well by adding 200 µL of a 5×10^4 cells/mL suspension to each well of a 96-well tissue culture plate and allowed to grow for 24 h before treatment. The number of cells was assessed by trypan blue dye exclusion.

The stock solution of sinapic acid was freshly prepared in PBS with 50% DMSO and filtered with Millipore filters (0.20 µm). A working solution in the related culture medium was used. The final DMSO concentration in medium was 0.5% (v/v). The cells were treated with sinapic acid within a range of concentrations from 0 to 20000 µM in the culture medium for 18 h. Control experiments were carried out with the culture medium containing 0.5% of DMSO without sinapic acid.

The cytotoxicity of sinapic acid was carried out in V79 and HeLa cells by neutral red uptake assay (NRU) assay following the protocols described by Virgilio et al. (16) and Saquib et al. (17). It is based on the determination of the accumulated neutral red (NR) dye in the lysosomes of viable cells.

Following the treatment, the cells were incubated for 3 h with NR dye (50 µg/mL) dissolved in serum free medium. Cells were then washed with PBS three times. After removing any remaining liquids from the wells, the cells were fixed with 200 µL of NR fixative solution (ethanol: acetic acid: water, 50:10:40, v/v/v) followed by shaking for 20 min at 600 rpm. Absorbance of each sample was measured at 540 nm using the microplate reader (SpectraMax M2, Molecular Devices Limited, Berkshire, UK). Cell viability was calculated as percent survival, determined by the number of treated over control cells X 100 (% cell viability). IC_{50} values of sinapic acid, the concentration reducing the absorbance of treated cells by 50% with reference to the control (untreated cells), were determined from the dose-response curves.

Statistical analysis

The statistical analysis was performed using the software programs SPSS 15.0 (SPSS, Chicago, IL, USA). All experiments were carried out in quadriplicate. The distribution of the data was checked for normality using the Shapiro-Wilk test. The homogeneity of the variance was verified by the Levene test. Differences between the means of data were compared by the one way variance analysis test and post hoc analysis of group differences was performed by least significant difference test. The results were given as the mean ± standard deviation. *P* value of less than 0.05 was considered as statistically significant.

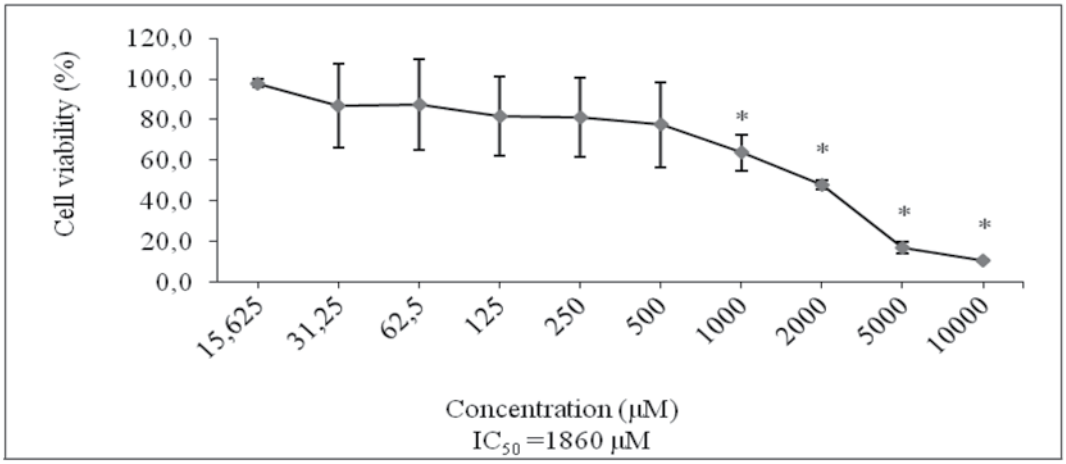


Figure 1. Effects of sinapic acid on V79 cells viability in NRU assay.
The results were expressed as percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as percent of control (assuming data obtained from absence of sinapic acid as 100%). NRU: neutral red uptake. Results were given as the mean ± standard deviation.* $p<0.05$, compared to negative control.

RESULTS

The cytotoxic effects of different concentrations of sinapic acid on V79 cells as measured by the NRU assay have been shown in Figure 1. V79 cell line was chosen because of its high sensitivity to various chemicals, high cloning efficiency, and excellent properties in colony formation. According to

the results, compared to the same concentrations of untreated cells (control group); sinapic acid was found to have cytotoxic effects in concentrations higher than 2000 µM. A concentration dependent toxicity was observed in V79 cells after 18 h exposure to sinapic acid.

The concentrations up to 500 µM had no significant effect on V79 cell viability during 18 h exposure. IC_{50} value of sinapic acid in V79 cell line was found to be 1860 µM (Figure 1).

The cytotoxic effects of the different concentrations of sinapic acid on HeLa cells as measured by the NRU assay has been shown in Figure 2. According to the results, compared to the same concentrations of untreated cells (control group); sinapic acid was found to have cytotoxic effects in concentrations higher than 5000 µM. A concentration dependent toxicity was observed in HeLa cells after 18 h exposure to sinapic acid.

The concentrations up to 2000 µM had no effect on HeLa cell viability during 18 h exposure. IC_{50} value of sinapic acid in HeLa cell line was found to be 7248 µM (Figure 2).

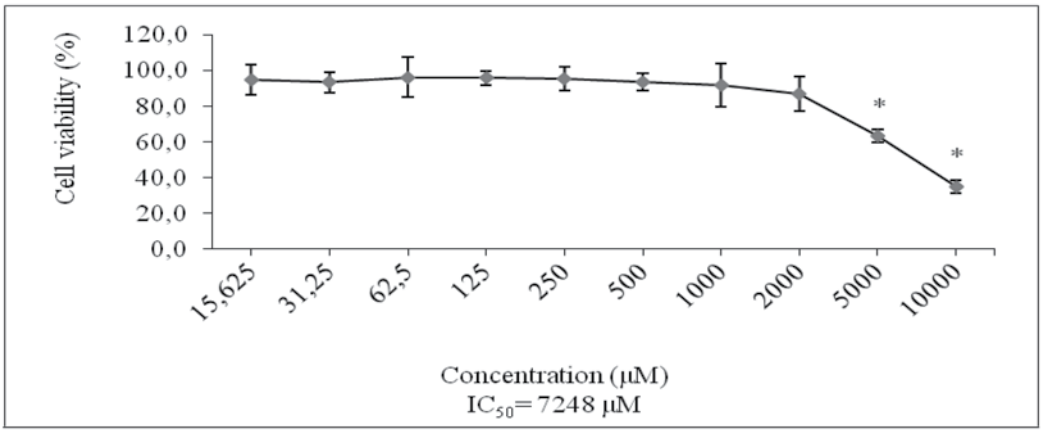


Figure 2. Effects of sinapic acid on HeLa cells viability in NRU assay.
The results were expressed as percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as percent of control (assuming data obtained from absence of sinapic acid as 100%). NRU: neutral red uptake. Results were given as the mean ± standard deviation.* $p<0.05$, compared to negative control.

DISCUSSION

Phenolic compounds play an important role in the prevention of various degenerative disorders or diseases related to oxidative damage due to their antioxidant properties (18). The antioxidant activity of phenolic compounds depends on the structure, in particular the number and the positions of the hydroxyl groups and the nature of substitutions on the aromatic rings (4-6).

Sinapic acid is a small naturally occurring hydroxycinnamic acid derivative. It is a phenolic compound and a member of the phenylpropanoid family, which are assumed as therapeutically beneficial and generally not toxic. Sinapic acid is widespread in the plant kingdom (fruits, vegetables, cereal grains, oilseed crops, and some spices and medicinal plants) and is common in the human diet (9). However, some reports on the properties of sinapic acid showed that these compounds might be considered for potential use as preservatives in foods, cosmetics, and in the pharmaceutical industry (19,20).

Sinapic acid has been suggested to show antioxidant, antimicrobial, anti-inflammatory, anticancer, and anxiolytic activity (9). On the other hand, there are very few studies related to the cytotoxicity of sinapic acid. The cytotoxicity of sinapic acid has been studied in human neuroblastoma cells (SH-SY5Y) by MTT assay. The cells were incubated with different concentrations of sinapic acid (0, 1, 10, and 100 µg/mL) for 24 h. The results showed that sinapic acid did not induce cytotoxic effect on SH-SY5Y cells at the concentrations studied (21). In an another

study by Zhang et al. (22), the cytotoxic effect of sinapic acid was also tested on human neuroblastoma (SH-SY5Y) cells at different concentrations for 24 h by using the 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) assay. Sinapic acid did not affect the viability of SH-SY5Y within the concentration range used (0–20.0 μM) and the EC_{50} (half maximal effective concentration) of sinapic acid in this study was found to be 1.96 ± 0.23 μM. In this study, sinapic acid exhibited moderate protection against H_2O_2-induced cell death in dose dependent manner. Balaji et al. (23) studied the effect of sinapic acid on the viability of colon cancer cell lines (HT-29 and HSW480) using MTT assay at different time point and concentrations. Sinapic acid was found to inhibit the viability of colon cancer cell lines in a dose and time dependent manner with IC_{50} values of 138.11 μg/mL (HT-29) and 112.55 μg/mL (SW480) for 24h incubation. Janakiraman et al. (24) also investigated the cytotoxic effect of sinapic acid (25-250 μM) for 24 and 48 hr in human laryngeal carcinoma cell line (HEp-2) by MTT assay. The IC_{50} values of sinapic acid were found to be 125.23 μM for 24 hr and 117.81 μM for 48 hr. In our study, the cytotoxic effects of sinapic acid in HeLa cells occurred in concentrations higher than 5000 μM and IC_{50} value of sinapic acid was 7248 μM for 18 treatments by NRU assay. The concentrations up to 2000 μM had no effect on HeLa cell viability for 18 h. We suggest that Sinapic acid has different cytotoxicity profile according to the cell type, treatment time and cytotoxicity method used.

The effectiveness of sinapic acid as pBR322 plasmid DNA-cleaving agents in the presence of Cu^{2+} ions was investigated. Sinapic acid was remarkably more effective at causing DNA damage than other phenolic compounds toward human promyelocytic leukemia (HL-60) cell proliferation. Addition of exogenous Cu^{2+} ions resulted in dichotomy on cell viability depending on the concentration of sinapic acid, that is, low concentrations of sinapic acid enhanced the cell viability, and conversely, high concentrations of sinapic acid almost completely inhibited the cell proliferation. The good correlation between the DNA damaging activity and the oxidative potential of the sinapic acid indicates that the electron transfer between HCAs and Cu^{2+} plays a crucial role in the reaction (25, 26).

Antiproliferative and apoptotic effects of sinapic acid was examined in human breast cancer cells (T47D). Sinapic acid was found to be only partial inhibitor to cell growth, decreasing cell proliferation with IC_{50} of 7×10^{-11} M. It was also reported that the inhibitory and pro-apoptotic effects of sinapic acid on tumoral proliferation might be due to its direct interaction with the aryl hydrocarbon receptor, the nitric oxide synthase inhibition and its pro-apoptotic effect (27).

In our study, the cytotoxicity of sinapic acid using the NRU assay in V79 and HeLa cells which are high sensitivity to various chemicals, high cloning efficiency, and excellent properties in colony formation, was investigated. Our results demonstrated that, at the concentrations up to 500 μM, sinapic acid had no effect on V79 cell viability during 18 h exposure but at concentrations higher than 1000 μM, the cell viability decreased below 50 % and IC_{50} value of sinapic acid in V79 cell line was found to be 1860 μM. At the concentrations up to 2000 μM, sinapic acid had no effect on HeLa cell viability during 18 h exposure but at concentrations higher than 5000 μM, the cell viability decreased below 50 %. IC_{50} value of sinapic acid in HeLa cell line was found to be 7248 μM.

CONCLUSION

In conclusion, the results of this study suggest that sinapic acid might have different cytotoxic effects in different cell lines in a dose dependent manner, but in general it is shown that sinapic acid has no cytotoxic effects in the studied cell lines except at very high concentrations. Having many useful pharmacological properties of sinapic acid, it must be considered that sinapic acid may have different cytotoxic effects on different cell lines. Therefore, the further *in vitro* and *in vivo* studies should be performed to bring out the toxic/beneficial effects of sinapic acid and its derivatives for safety uses.

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