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Antioxidant capacities and cytotoxic properties of some natural phenolic compounds in different cell lines

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Merve Bacanlı*, Hatice Gül Göktaş*,, Zehra Sarıgöl*, Sevtap Aydın*, A. Ahmet Başaran***, Nurşen Başaran*°**

Introduction

Oxidative stress which involved in the initiation and/or progression of several diseases such as inflammatory injury, ageing processes, cancer, atherosclerosis, rheumatoid arthritis, neurodegenerative and cardiovascular diseases is the state of imbalance between the level of antioxidant defence system and production of reactive oxygen species (ROS). ROS include a number of chemicals derived from oxygen such as superoxide radical, hydrogen peroxide, nitric oxide and hydroxyl radical[1]. The major cellular targets of ROS are membrane lipids, proteins, nucleic acids and carbonhydrates[2]. Under normal conditions, the balance between production and elimination of free radicals is maintained by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) and other sources such as some metals, glutathione (GSH), vitamins and phytochemicals[3]. Recent studies have shown that antioxidants are capable of protecting cells from oxidative damage[4].

Natural products are widely being used as dietary supplements for health preventing effects because of their potential antioxidant properties[3,5]. Plant polyphenols may act as antioxidants by different mechanisms such as free radical scavenging, metal chelation and protein binding. Often, more than one mechanism is involved, therefore causing synergism[6,7]. Furthermore,

* Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey
**Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Cukurova University, Adana, Turkey
***Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey
° Corresponding author: e-mail address: nbasaran@hacettepe.edu.tr

other beneficial effects of natural antioxidants have been reported such as antibacterial, antiviral, antimutagenic, antiallergic, anticarcinogenic, antihypertensive and antiulser[8,9,10,11]. Nevertheless, there are still many phenolic compounds with unclear or unidentified prooxidant and antioxidant properties[12].

The aim of this study was to evaluate the antioxidant capacity of three commonly using phenolic compounds (curcumin, resveratrol and rosmarinic acid) by the trolox equivalent antioxidant capacity (TEAC) assay and their cytotoxicity by neutral red uptake (NRU) assay in Chinese Hamster Ovary (CHO), Human Breast Carcinoma (BT-474) and Human Epithelial Adenocarcinoma (HeLa) cells.

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), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid, ABTS$^{\bullet}$), potassium peroxodisulfate ($K_2S_2O_8$), 6-hydroxy-2,5,7,8-tetramethylchromon-2-carboxylic acid (trolox) (purity >97%), curcumin, resveratrol and rosmarinic acid from Sigma (St Louis, USA).

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

A wide range of methods have been currently used to assess the antioxidant capacity[13]. TEAC assay, the most popular antioxidant activity screening method, described by Miller et al. (1993), is based on scavenging of long-lived, stable blue/green radical (ABTS$^{\bullet}$), converting it into a colorless product[14]. The degree of decolorization gives the antioxidant capacity and reflects the amount of ABTS$^{\bullet}$ that has been scavenged and can be determined spectrophotometrically at 734 nm[15].

ABTS$^{\bullet}$ was produced by reacting 14 µM ABTS stock solution with 4,9 mM $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at + 4 °C for 12–16 h before use. The ABTS$^{\bullet}$ solution was diluted with ethanol to give an absorbance of 1.4 (±0.05) at 734 nm. After addition of 500 µl of diluted ABTS$^{\bullet}$ solution to 500 µl of antioxidant compound solutions or trolox standards (at concentrations 2, 2.5, 5, 7.5, 10, 25, 50, 100 and 200 µM) in ethanol, the absorbance was read after 1 minute of initial mixing.

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 with CHO, BT-474 and HeLa cell lines by NRU assay following the protocols described by Virgilio et al. (2004) and Saquib et al. (2012)[16,17].

Following disaggregation of cells with trypsin/EDTA and resuspension of cells in medium, a total of 10^5 cells/well were plated in 96-well tissue-culture plates. After 24 h incubation, the different concentrations (0-400 μM) of curcumin, resveratrol and rosmarinic acid in medium were added. The cells were incubated for 18 h (1.5 cell cycle) at 37°C in 5% CO_2 in air, then the medium was aspirated. The cells were washed twice with PBS and incubated for an additional 3 hours 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 percent of control (assuming data obtained from the absence of phenolic compounds as 100 %). IC_{50} values represent the concentrations that reduced the mean absorbance of 50% of those in the untreated cells.

Statistical Analysis

Determinations of all samples were carried out in triplicate. Statistical analysis was performed by SPSS for Windows 20.0 computer program for TEAC assay. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences was performed by least significant difference (LSD) test. The results were given as the mean±standard deviation. p values of less than 0.05 were considered as statistically significant.

Results

Cytotoxicity

Cytotoxicity in CHO Cells

A concentration-dependent decrease was seen in the survival of cells exposed to curcumin, resveratrol and rosmarinic acid (Fig. 1). IC_{50} values of curcumin, resveratrol, and rosmarinic acid in CHO cells were found to be 50 µM, 120 µM and 150µM, respectively.

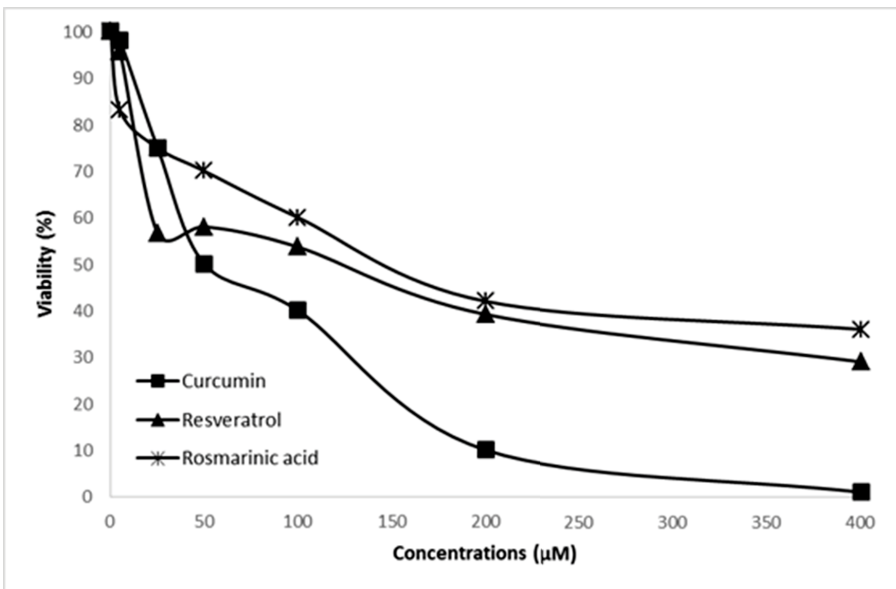


Figure 1
Effects of phenolic compounds on cell viability (%) in CHO cells.

Cytotoxicity in HeLa Cells

A concentration-dependent decrease was seen in the survival of HeLa cells exposed to curcumin. But resveratrol and rosmarinic acid did not seem to affect the survival of HeLa cells in the concentration studied (Fig. 2). Therefore, in HeLa cells, IC50 values of curcumin was found to be 48 µM whereas IC50 values of resveratrol and rosmarinic acid could not be calculated in the concentrations studied.

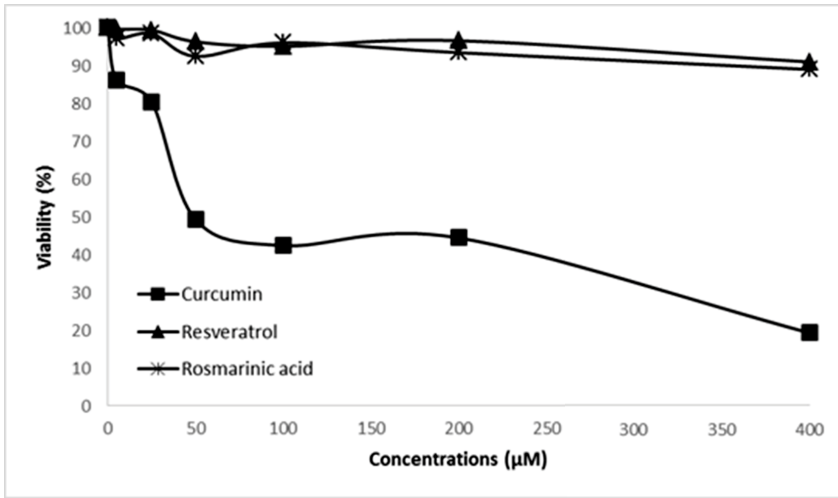


Figure 2
Effects of phenolic compounds on cell viability (%) in HeLa cells.

Cytotoxicity in BT-474 Cells

Although a concentration-dependent decrease was seen in the survival of BT-474 cells exposed to curcumin, resveratrol and rosmarinic acid in the concentrations studied, the phenolic compounds were not found to be cytotoxic hence the IC_{50} values were not calculated (Fig. 3).

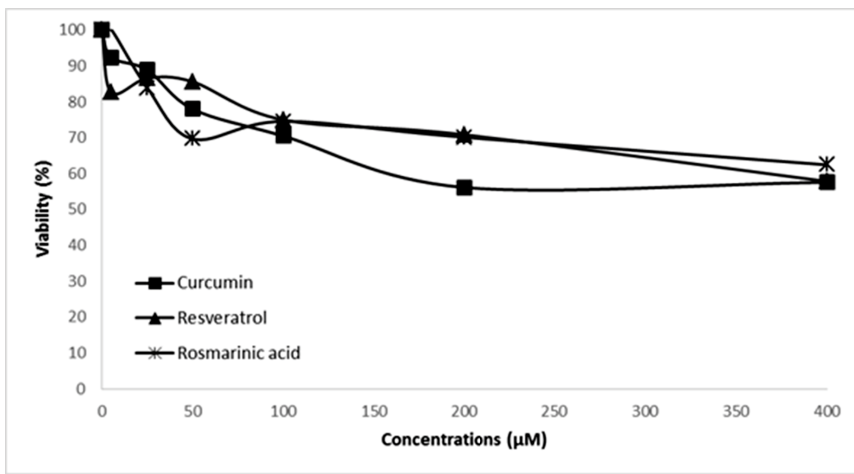
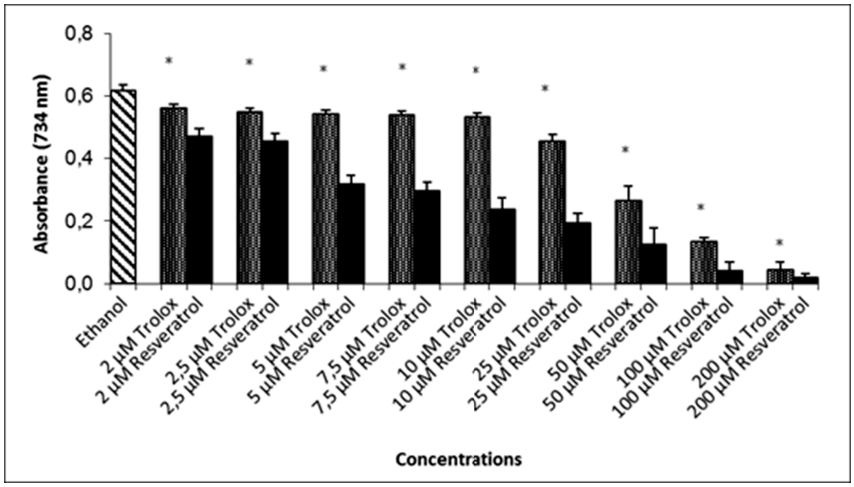
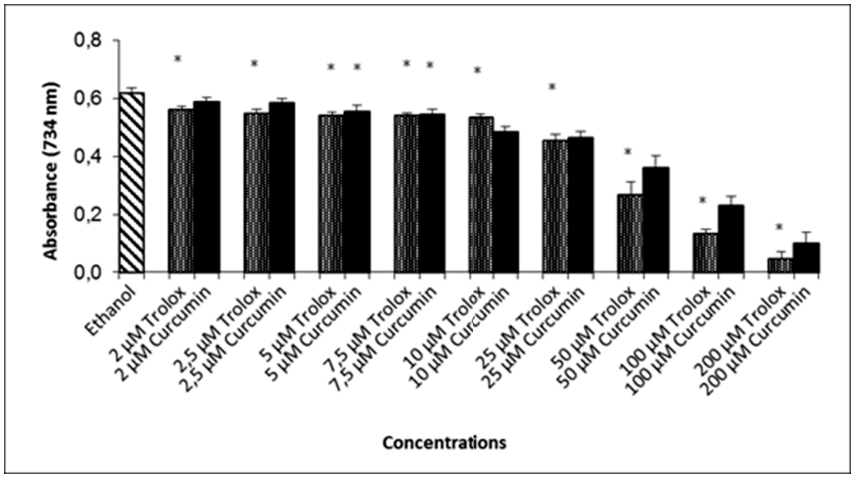


Figure 3
Effects of phenolic compounds on cell viability (%) in BT-474 cells.

Antioxidant Capacity

The antioxidant activities of each phenolic compounds at different concentrations were done (Figure 4). When compared to the same concentrations of reference antioxidant trolox, resveratrol and rosmarinic acid had significantly more antioxidant activity whereas curcumin had less antioxidant activity than trolox. Comparision of the antioxidant capacities of curcumin, resveratrol and rosmarinic acid to trolox were shown in Figure 5.





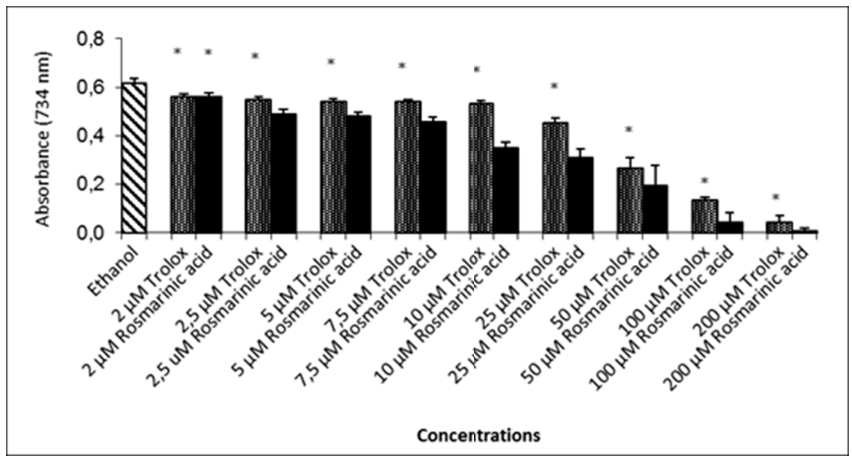


Figure 4

Antioxidant activities of each phenolic compound in relation to antioxidant trolox. Values were given as the mean± standard deviation $p<0.05$, significantly different from blank (ethanol).

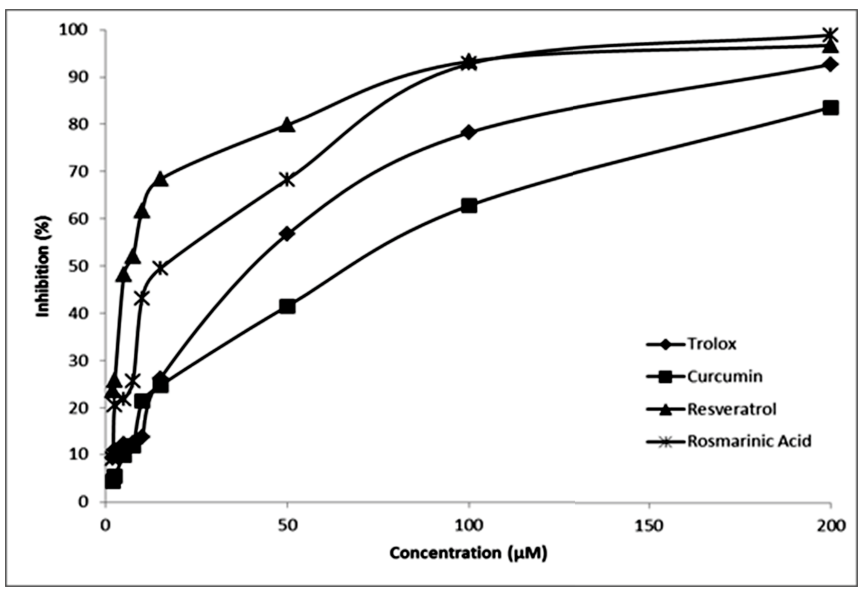


Figure 5

Comparision of the atioxidant capacities of curcumin, resveratrol and rosmarinic acid to trolox.

Discussion and Conclusion

Several studies have shown that fruits and vegetables rich diets can be associated with a markedly decreased risk of chronic diseases. This can be attributed to high levels of antioxidant compounds in these foods. Curcumin (1,7-bis(4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3,5-dione) is a polyphenolic yellow pigment, isolated from the rhizomes of *Curcuma longa* (turmeric), widely used in traditional medicine and as a spice in cooking [18-20]. It possesses anti-inflammatory, antibacterial, antiamyloid, anticancer and antioxidant activities[21-24]. The antioxidant activity of curcumin arises mainly from scavenging of several biologically relevant free radicals[25, 26]. Khopdea et al. (1999) have determined antioxidant activity of curcumin by two methods (TEAC and measurement of its ability to inhibit lipit peroxidation induced by ferric-ascorbat) and their results showed that curcumin was more effective than trolox[20]. Besides, Ak et al. (2008) have found that curcumin has more antioxidant capacity when compared to standard antioxidant compounds (butyl hydroxy anisole, butyl hydroxy toluene and trolox) in different *in vitro* assays (DPPH and TEAC assays)[27]. In this study, we assessed the antioxidant capacity of curcumin by TEAC assay, but it had less antioxidant activity at all tested concentrations (except 10 µM) than well known antioxidant trolox. Different *in vivo* and *in vitro* studies have reported that curcumin could inhibit the growth of various cancer cells from different organs. Lantto (2009) et al. studied cytotoxicity of curcumin in two different cell lines (neuroblastoma (SH-SY5Y) and fibroblast (CV1-P) cells) by MTT and LDH assays and their results indicated that curcumin significantly decreased the metabolic activity of these cells[28]. Also, Mehta (1997) et al. showed anti-proliferative effect of curcumin human breast tumor cell lines BT-20, T-47D, SKBR3 and MCF- 7 by MTT assay[29]. The effects of curcumin on the viability of human leukemia cell lines (U937 and Molt4) by MTT assay were also determined and cytotoxic effects of curcumin in these cell lines were shown to be concentration dependent[30]. In our study, we determined cytotoxic effects of curcumin in CHO, HeLa and BT-474 cell lines by NRU. Curcumin had more cytotoxic effect on CHO and HeLa cells compared to BT-474 cell line. These differences of cellular responces to curcumin might be arise from different metabolic pathways or receptors in the cells. Above the concentration of 50 µM curcumin seemed to have the cytotoxicity both in healthy cell line (CHO) and human epithelial adenocarcinoma (HeLa) cells but it did not show cytotoxic effects on human breast carcinoma cells (BT-474) at these concentrations.

Resveratrol (3,5,4'-trihydroxy-transstilbene), a naturally occurring phytoalexin, is present in grapes and several other common foodstuffs[31]. It is

also suggested to show various biological activities such as cardio protective, antiplatelet, anti-inflammatory, neuroprotective and antiviral[32,33]. Recently, Gülçin (2010) has clarified antioxidant and radical scavenging activities of resveratrol by different *in vitro* assays (DPPH, ABTS, DMPD, O_2, H_2O_2 scavering activity and total antioxidant activity) at 10, 20, 30 µg/mL concentrations[34]. Although in a very recent study, Xiang et al. (2014) showed that there was no difference in antioxidant activities between red wine and the red wine enriched 10-fold of resveratrol. Besides, they have claimed resveratrol did not affect directly antioxidant behavior of wine[35]. But in this study, the antioxidant capacities of resveratrol at even a wide concentration range (2, 2.5, 5, 7.5, 10, 25, 50, 100, 200 µg/mL) were found to be significantly more than trolox. Recently, Zhao et al. studied preventive effects of resveratrol in cancer cell lines (HeLa, MCF-7 and human APL NB4 cells) with MTT assay and they have indicated that there was a concentration-dependent cell growth inhibition rate after treatment with resveratrol in these cell lines[36]. In this study, we found that healthy CHO cell line were sensitive to cytotoxic effects of resveratrol but in contrasting with the literature, in cancer cell lines, cytotoxic activity of resveratrol was less than healthy cell line.

Rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenillactic), a potent antioxidative polyphenol, is distributed in Lamiaceae herbs[37]. It is widely used as a food additive and herbal tea. It has been suggested to have beneficial properties which include anti-inflammatuar, anti-mutagenicity, reduction of atopic dermatitis, photo protection of keratinocytes, protection of Alzheimer's disease and cancer[38]. Its antioxidant activity was indicated several *in vitro* and *in vivo* studies[39, 40]. In addition to that, it is reported to have prooxidant activity which is suggested to cause antiviral, anti-inflammatory and anti-microbial effects by generating ROS[41]. We found that rosmarinic acid had significantly more antioxidant capacity when compared to trolox. In literature, cytotoxic activity of rosmarinic acid is also contradictory and depends on cell lines. Makino et al. (2000) showed that rosmarinic acid had cytotoxic activity on murine mesangial cells in 24 h incubation by LDH and MTT assay[42]. On the other hand, Çeliktaş et al. (2010) indicated that rosmarinic acid had proliferative effects rather than cytotoxic activity in all cell lines tested; NCI-H82 (human small cell lung carcinoma), DU-145 (human prostate carcinoma), Hep-3B (human hepatocellular carcinoma), K-562 (human chronic myeloid leukemia), MCF-7 (human breast adenocarcinoma), PC-3 (human prostate adenocarcinoma) and MDA-MB-231 (human breast adenocarcinoma) with MTT assay and Moon et al. (2010) also reported that rosmarinic acid alone exhibited little effect on the cell viability in U-937 cell[43]. In the present study, we examined cytotoxicity of RA in two cancer cell lines (HeLa and BT-474)

and healthy cell line (CHO) by NRU assay. In agreement with the recent studies, according to the our results, rosmarinic acid showed cytotoxic activity in CHO cell line than HeLa and BT-474 cell lines.

In conclusion, in this study the antioxidant capacities and cytotoxic properties of curcumin, resveratrol and rosmarinic acid were examined. We found differences in the antioxidant capacity between these phenolic compounds being resveratrol and rosmarinic acid more active than curcumin. Also differences in the cytotoxicity of these plant phenolics in different cell lines were observed assuming that attention must be given in the usage of phenolic compounds in different disorders especially in different cancer types. Further investigation such as using more cell lines and more cytotoxicity assays and incubations with various concentrations at many time points should be performed to confirm beneficial and toxic effects of phenolics.

Summary

Plant phenolic compounds are important constituents of the human diet and in recent years attention has been drawn to beneficial properties of plants and its phenolic compounds. They exhibit a wide range of biological effects, including antioxidant, antiplatelet, anti-inflammatory and anticancer activities. Curcumin, resveratrol and rosmarinic acid are phenolic compounds which are known as antioxidants and commonly used for the preventation and treatment of oxidative stress related diseases such as cardiovascular and neurodegenerative disorders and cancer. In this study, we determined the antioxidant capacities of these phenolic compounds by the trolox equivalent antioxidant capacity (TEAC) assay and their cytotoxicity by neutral red uptake (NRU) assay in healthy cell line (i.e., chinese hamster ovary (CHO)), and two tumor cell lines (human breast carcinoma (BT-474) and human epithelial adenocarcinoma (HeLa)). Our results showed that resveratrol and rosmarinic acid have significantly more antioxidant capacity than trolox whereas curcumin showed less antioxidant capacity compared to trolox. In healty CHO cell line, curcumin, resveratrol and rosmarinic acid showed significantly cytotoxic activity, but in cancer cell lines such as HeLa and BT-474, all of the tested compounds have shown different profile. It seems to be not cytotoxic to HeLa and BT-474 cancer cell lines assuming that its usage in these cancer types are not beneficial.

Keywords: Curcumin, Resveratrol, Rosmarinic acid, Cytotoxicity, Trolox equivalent antioxidant capacity assay, Neutral red uptake assay.

Özet

Bazı Doğal Fenolik Bileşiklerin Antioksidan Kapasiteleri ve Farklı Hücre Hatlarında Sitotoksik Etkileri

Bitkisel fenolik bileşikler insan beslenmesinin önemli bir bileşenidir ve son yıllarda bitkiler ve bitkisel fenolik bileşiklerin yararlı özelliklerine olan ilgi yoğunlaşmıştır. Bu bileşikler antioksidan, antiplatelet, antienflamatuvar ve antikanser etkiler gibi çok çeşitli biyolojik etkiler göstermektedirler. Kurkumin, resveratrol ve rosmarinik asit antioksidan etkileri bilinen fenolik bileşiklerdir ve genellikle kardiyovasküler ve nörodejeneratif hastalıklar gibi oksidatif stres ile ilişkili hastalıkların tedavisinde ve bu hastalıklardan korunmada kullanılmaktadırlar. Bu çalışmada, bu fenolik bileşiklerin antioksidan kapasiteleri troloks eşdeğer antioksidan kapasite yöntemi (TEAC) ile ve sitotoksisiteleri sağlıklı hücre hattı (Çin hamster yumurtalık hücreleri CHO) ve tümor hücre hatlarında (İnsan meme kanseri hücreleri (BT-474) ve insan epitelyal adenokarsinom hücreleri (HeLa)) nötral kırmızı alım yöntemi ile belirlenmiştir. Bu çalışmanın sonucunda, resveratrol ve rosmarinik asitin trolokstan çok daha fazla antioksidan kapasiteye sahip olduğu, kurkuminin ise troloksa göre daha az antioksidan kapasiteye sahip olduğu gösterilmiştir. Sağlıklı CHO hücre hattında, kurkumin, resveratrol, rosmarinik asitin önemli sitotoksik etkileri olduğu görülmüştür. Ancak HeLa ve BT-474 gibi kanser hücrelerinde, bu bileşiklerin farklı özellik göstererek bu hücrelerde sitotoksik olmadıkları ve bu nedenle, bu kanser türlerinde kullanımlarının faydalı olamayacağı görülmüştür.

Anahtar kelimeler: Kurkumin, Resveratrol, Rosmarinik asit, Sitotoksisite, Troloks eşdeğer antioksidan kapasite testi, Nötral kırmızı alım testi.

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