Assessment of Cytotoxicity Profiles of Different Phytochemicals: Comparison of Neutral Red and MTT Assays in Different Cells in Different Time Periods

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ABSTRACT

Objectives: Phenolic compounds exhibit several health protective properties. Galangin, curcumin, pycnogenol, puerarin and ursolic acid are commonly used plant phenolics in folk medicine. The aim of our study was to evaluate the difference between neutral red uptake (NKA) and MTT assays using different plant phenolics (galangin, curcumin, pycnogenol, puerarin and ursolic acid) in healthy and cancer cells in different time periods.

Materials and Methods: In this study, the cytotoxic effects of these phenolic compounds were investigated by NRU and MTT assays in healthy (V79, Chinese hamster fibroblast cell line) and cancer [human cervix epithelial adenocarcinoma cell line Henrietta Lacks (HeLa) and human mammary carcinoma cell line (BT-474)] in 18, 24 and 48 h incubation periods.

Results: Our results demonstrated that galangin, curcumin, pycnogenol, puerarin and ursolic acid decreased cell viability of V79, HeLa and BT-474 cells in a dose-dependent manner in 18, 24 and 48 h incubation periods. However, the cell survival rate was much lower in 48 h incubation period. There was no difference between the results from NRU and MTT assays.

Conclusion: To decide which incubation period and which cytotoxicity study to be used, the cytotoxicity mechanism of the compound must be known.

Key words: MTT, neutral red, plant phenolics

ÖZ

Amaç: Fenolik bileşikler sağlığı koruyucu farklı özellikliler gösterir. Galangin, kurkumin, pycnogenol, puerarin ve ursolic asit halk tıbbında yaygın olarak kullanılan bitkisel fenoliklerdir. Bu çalışmanın amacı, nötral kırmızı alm (NKA) ve MTT yöntemleri arasındaki farkı sağlıklı hücreler ve kanser hücrelerinde aynı zaman aralıklarında belirlemektedir.

Gereç ve Yöntemler: Bu çalışmada, fenolik bileşiklerin sitotoksisite etkileri sağlıklı hücreler (Çin hamster fibroblast hücre hattı) ve kanser hücreleri (insan serviks epitelyal adenokarsinoma hücre hattı Henrietta Lacks (HeLa) ve insan meme karsinoma hücre hattı (BT-474)) öncesinde 18, 24 ve 48 saatlik inkübasyon sürelerinde NKA ve MTT yöntemleriyle değerlendirilmiştir.

Bulgular: Bulgularımız galangin, kurkumin, pycnogenol, puerarin ve ursolic asit V79, HeLa ve BT-474 hücre canlılıklarını 18, 24 ve 48 saatlik inkübasyon sürelerinde aynı zaman aralıklarında farklı şekilde eleyici etkiler oluşturmuş, ancak en az hücre canlanması oranı 48 saatlik inkübasyon sonrası görülmemiştir. NKA ile MTT yönteminin sonuçları arasında fark görülmemiştir.

Sonuç: Sitotoksisite analizinde kullanılan yöntem ve inkübasyon süresinin belirlenmesi için maddelerin sitotoksisite mekanizması bilinmelidir.

Anahtar kelimeler: MTT, nötral kırmızı, bitkisel fenolikler
INTRODUCTION

Consumption of great amounts of fruits and vegetables rich in phenolic compounds has been associated with health benefits such as anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, and cardioprotective effects. Due to the cytotoxicity profile of many phenolic compounds, it is suggested that these compounds 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), is present at high concentrations in propolis and in an Indian root, Alpinia officinarum, which is a common spice in Asia. It is suggested that galangin has antioxidant, antimutagenic, anti-inflammatory, antiviral and anticancer properties.

Curcumin (diferuloyl methane), the major yellow pigment from the rhizomes of turmeric (Curcuma longa L.), have gained increasing interest because of its chemopreventive properties against human cancers. Turmeric, the powdered rhizome is commonly used as an antiseptic, antidote for poisoning, for treating respiratory disorders, some skin diseases, and as a household remedy for treating sprains and swellings caused by injury.

Pycnogenol (PYC) is a standardized natural plant extract obtained from the bark of the French maritime pine Pinus pinaster (formerly known as Pinus maritime). PYC has been used in European countries as a dietary food supplement. It has strong antioxidant activity and capacity to efficiently scavenge reactive oxygen and nitrogen species.

Puerarin (daidzein-8-C-glucoside) is the main isoflavone derived from the root of Pueraria lobata (kudzu root). In experimental models it is also suggested to be used in the prevention and treatment of cardiovascular diseases, diabetes, cancer and osteoporosis.

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenoid obtained from plants. It has long been used in traditional Chinese medicine because of its anti-inflammatory, anti-artheritic, cytostatic and anti-proliferative, hepatoprotective effects.

Cytotoxicity assays are widely used in toxicity studies. The NR uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays are commonly used cytotoxicity assays to determine the cytotoxic properties of compounds. NRU assay has been used as an indicator of cytotoxicity in cultures of primary hepatocytes and other cell lines. Living cells take up the neutral red, which is concentrated within the lysosomes of cells. MTT, a water soluble tetrazolium salt, is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells.

The aim of our study was to evaluate the difference between NRU and MTT assays using different plant phenolics (galangin, curcumin, PYC, puerarin and ursolic acid) in healthy (V79, Chinese hamster fibroblast cell line) and cancer (human cervix epithelial adenocarcinoma cell line (HeLa) and human mammary carcinoma cell line (BT-474)) cells in different time periods (8, 24 and 48 h).

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, Triton X-100, phosphate buffered saline (PBS), ethanol, NR, MTT, galangin, curcumin (97%, purity), ursolic acid from Sigma (St Louis, USA), puerarin from Fluka (St. Gallen, Switzerland). PYC®, a registered trade mark of Horphag Research Ltd., (Geneva, Switzerland), was provided by Henkel Corporation (La Grange, IL, U.S.A.).

Cell culture

V79, HeLa and BT-474 cells were seeded in 75 cm² flasks in 20 mL MEM supplemented with 10% FCS and 1% penicillin-streptomycin and then grown for 24 h in an incubator at 37°C in an atmosphere supplemented with 5% CO₂.

Determination of cytotoxicity by NRU assay

The cytotoxicity of phenolic compounds was performed in V79, HeLa and BT-474 cell lines by NRU assay following the protocols described by Di Virgilio et al. and Saquib et al. Following disaggregation of cells with trypsin/EDTA and resuspension of cells in the medium, a total of 10⁵ cells/well were plated in 96 well tissue-culture plates. After 24 h incubation, the different concentrations of galangin, curcumin, PYC, puerarin and ursolic acid in medium were added. The cells were incubated for 18, 24 and 48 h at 37°C in 5% CO₂, then the medium was aspirated. The cells were then incubated for an additional 3 h in the medium supplemented with NR (50 μg/mL). 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%).

Determination of cytotoxicity by MTT assay

MTT assay was performed by the method of Mosmann with the modifications of Holst-Hansen and Brünner and Kuzma et al. A total of 10⁵ cells/well were plated in 96 well tissue-culture plates. After 24 h incubation, cells were exposed to the different concentrations of galangin, curcumin, PYC, puerarin and ursolic acid in medium for 18, 24 and 48 h at 37°C in 5% CO₂ in air. Then, the medium was aspirated and MTT (5 mg/mL of stock in PBS) was added (10 μL/well in 100 μL of cell suspension), and cells were incubated for an additional 4 h with MTT dye. At the end of incubation period, the absorbance of the solution in each well was measured in a microplate reader at 570 nm. Results were expressed as the mean percentage of cell growth from three independent experiments. Cell viability was...
plotted as the percent of control (assuming data obtained from the absence of phenolic compounds as 100%).

RESULTS

Determination of cytotoxicity in V79 cell line
A concentration dependent decrease was seen in the survival of cells exposed to galangin, curcumin, PYC, puerarin and ursolic acid in all time periods in both cytotoxicity assays. But in 48 h incubation period, the cell survival is found much lower (Table 1) (Figure 1, 2).

Determination of cytotoxicity in HeLa cell line
A concentration dependent decrease was seen in the survival of cells exposed to galangin, curcumin, PYC, puerarin and ursolic acid in all time periods in both cytotoxicity assays. But in 48 h incubation period, the cell survival is found much lower (Table 2) (Figure 3, 4).

Determination of cytotoxicity in BT-474 cell line
A concentration dependent decrease was seen in the survival of cells exposed to galangin, curcumin, PYC, puerarin and ursolic acid in all time periods in both cytotoxicity assays. But in 48 h incubation period, the cell survival is found much lower (Table 3) (Figure 5, 6).

Figure 1. Cytotoxic effects of a) galangin, b) curcumin, c) pycnogenol, d) puerarin and e) ursolic acid in V79 cells by neutral red uptake assay.
DISCUSSION

The cytotoxic effects of galangin, curcumin, PYC, puerarin and ursolic acid were investigated by NRU and MTT assays in V79, HeLa and BT-474 cells in 18, 24 and 48 h incubation periods. This is the first study about cytotoxic effects of these phenolics in healthy and cancer cell lines with two different assays and different incubation periods. Our results demonstrated that both galangin, curcumin, PYC, puerarin and ursolic acid decreased cell viability of V79, HeLa and BT-474 cells in a dose dependent manner in 18, 24 and 48 h incubation periods. But the cell survival rate was much lower in 48 h incubation period.

In SNU-484 cells, galangin has shown cytotoxic effect in a dose dependent manner and IC50 value of galangin in this cell line has found 100 μM.22 In an another cytotoxicity study with galangin, it has shown that the cytotoxic effect has increased in a dose dependent manner on HepG2 cells.23 As a result of the small number of studies carried out that galangin has no cytotoxic activity under 100 μM in different methods and different cell lines. Lantto et al.24 have studied cytotoxicity of curcumin in two different cell lines [neuroblastoma (SH-SY5Y) and fibroblast (CV1-P) cells] by MTT and lactate dehydrogenase (LDH) leakage assays and their results have indicated that curcumin significantly decreased the metabolic activity of these cells.24 Also, Mehta et al.25 have showed anti-proliferative effect of curcumin on human breast tumor cell lines BT-20, T-47D, SKBR3 and MCF-7 by MTT assay. The effects of curcumin on the viability of human leukemia cell lines (U937 and Molt4) by MTT assay were also determined and dose dependent cytotoxic effects of curcumin were found.26 Taner et al.27 demonstrated the cytotoxic profile of PYC in healthy CHO cells. In this study, PYC has not showed cytotoxic effects at the concentrations of up to 150 μg/mL in CHO cells during 24 h exposure but above this concentration the cytotoxicity of PYC has started and the cell viability was decreased below 50% at 300 μg/mL.27 There is limited study about cytotoxicity of puerarin. In a single study, it is demonstrated that puerarin has shown cytotoxic effects on HT-29 cells in a dose and time dependent manner.28 In CaCo-2 cells, the viability of cells has decreased at concentrations higher than 100 μM with ursolic acid exposure for 48 h29,30 have demonstrated that ursolic acid decreased the cytotoxic effects of ultraviolet B on lymphocytes in trypan blue and MTT methods. It has been reported that different cytotoxicity assays can give
different results due to the chemical and the cytotoxicity assay employed. Fotakis and Timbrell\textsuperscript{16} have compared four different cytotoxicity assays (LDH, a protein, NRU and MTT assays). Different sensitivity was observed for each assay. The NRU and the MTT assays were found to be the most sensitive in detecting cytotoxic events. Putnam et al.\textsuperscript{32} have also studied cytotoxicity of cigarette smoke condensate with eight different (NRU, LDH release, kenacid blue binding, MTT, XTT, acid phosphatase activity, sulforhodamine B binding and resazurin binding) cytotoxicity assays. Four of the more widely used cytotoxicity assays (NRU, MTT, kenacid blue and LDH) were also evaluated at 3, 6, 12 and 18 h time points in this study. They have concluded that assays that measure membrane integrity (LDH) are useful for short exposure times (1 h), NRU assay was the most sensitive for moderate (3-6 h) exposure times; and assays that measure total cell number (NRU and kenacid blue) were more sensitive for longer exposure times (12, 18 and 24 h).\textsuperscript{32} But in our study, both phenolics showed similar cytotoxicity profile in NRU and MTT assays in all exposure times.

**Figure 3.** Cytotoxic effects of a) galangin, b) curcumin, c) pycnogenol, d) puerarin and e) ursolic acid in HeLa cells by neutral red uptake assay.
Figure 4. Cytotoxic effects of a) galangin, b) curcumin, c) pycnogenol, d) puerarin and e) ursolic acid in HeLa cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay.
Figure 5. Cytotoxic effects of a) galangin, b) curcumin, c) pycnogenol, d) puerarin and e) ursolic acid in BT-474 cells by neutral red uptake assay.
Figure 6. Cytotoxic effects of a) galangin, b) curcumin, c) pycnogenol, d) puerarin and e) ursolic acid in BT-474 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay.
Table 1. Viability (%) of V79 cells exposed to galangin, curcumin, pycnogenol, puerarin and ursolic acid

|                  | 18 h NRU (%) | 18 h MTT (%) | 24 h NRU (%) | 24 h MTT (%) | 48 h NRU (%) | 48 h MTT (%) |
|------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| **Negative control** | 100.00       | 100.00       | 100.00       | 100.00       | 100.00       | 100.00       |
| 1000 μM galangin  | 48.263       | 58.388       | 52.579       | 56.372       | 25.898       | 28.426       |
| 800 μM galangin   | 53.444       | 67.179       | 57.996       | 59.919       | 28.986       | 33.411       |
| 400 μM galangin   | 69.153       | 71.308       | 67.857       | 71.913       | 30.428       | 36.102       |
| 200 μM galangin   | 62.689       | 73.759       | 74.404       | 80.607       | 34.105       | 40.276       |
| 100 μM galangin   | 72.019       | 77.114       | 80.059       | 84.335       | 35.405       | 43.396       |
| 50 μM galangin    | 75.271       | 76.319       | 86.388       | 87.156       | 37.802       | 44.670       |
| 25 μM galangin    | 78.559       | 80.393       | 90.595       | 90.845       | 39.569       | 47.389       |
| 10 μM galangin    | 83.979       | 86.929       | 99.880       | 93.649       | 42.799       | 46.417       |
| 5 μM galangin     | 92.412       | 92.023       | 99.107       | 96.603       | 49.563       | 50.841       |
| 2 μM galangin     | 96.160       | 99.595       | 99.503       | 98.343       | 52.244       | 57.574       |
| 1000 μM curcumin  | 65.148       | 71.208       | 65.297       | 70.663       | 33.942       | 38.498       |
| 800 μM curcumin   | 68.951       | 69.821       | 66.726       | 71.680       | 34.044       | 42.232       |
| 400 μM curcumin   | 69.098       | 72.047       | 75.396       | 71.555       | 39.609       | 41.973       |
| 200 μM curcumin   | 75.950       | 76.251       | 81.527       | 80.240       | 46.008       | 43.764       |
| 100 μM curcumin   | 72.478       | 76.954       | 87.373       | 84.750       | 43.753       | 46.957       |
| 50 μM curcumin    | 79.845       | 79.184       | 85.158       | 86.513       | 48.263       | 47.626       |
| 25 μM curcumin    | 85.430       | 87.181       | 90.674       | 88.853       | 49.421       | 50.540       |
| 10 μM curcumin    | 92.100       | 89.417       | 94.246       | 89.146       | 51.635       | 53.518       |
| 5 μM curcumin     | 95.002       | 92.465       | 97.579       | 91.759       | 50.903       | 54.381       |
| 2 μM curcumin     | 95.241       | 98.774       | 99.503       | 96.821       | 52.447       | 56.517       |
| 1000 μM pycnogenol| 46.298       | 51.899       | 45.396       | 52.039       | 23.014       | 24.638       |
| 800 μM pycnogenol | 50.374       | 55.883       | 48.571       | 52.449       | 26.203       | 30.837       |
| 400 μM pycnogenol | 49.770       | 57.770       | 50.972       | 53.896       | 30.550       | 31.506       |
| 200 μM pycnogenol | 60.812       | 59.709       | 55.079       | 59.941       | 37.863       | 33.060       |
| 100 μM pycnogenol | 71.596       | 63.510       | 69.642       | 73.577       | 41.174       | 35.952       |
| 50 μM pycnogenol  | 77.916       | 66.986       | 73.948       | 85.599       | 43.956       | 38.261       |
| 25 μM pycnogenol  | 80.984       | 72.618       | 83.551       | 77.793       | 44.322       | 42.188       |
| 10 μM pycnogenol  | 85.118       | 77.989       | 85.436       | 86.266       | 46.597       | 46.310       |
| 5 μM pycnogenol   | 87.304       | 90.645       | 94.424       | 92.311       | 49.461       | 49.396       |
| 2 μM pycnogenol   | 95.352       | 99.739       | 97.420       | 92.460       | 51.046       | 49.979       |
| 1000 μM puerarin  | 50.560       | 59.885       | 50.521       | 55.571       | 27.117       | 29.802       |
| 800 μM puerarin   | 50.909       | 69.520       | 52.480       | 58.528       | 29.920       | 29.258       |
| 400 μM puerarin   | 63.935       | 77.557       | 56.805       | 60.408       | 29.839       | 30.082       |
| 200 μM puerarin   | 71.284       | 78.059       | 61.567       | 69.788       | 34.125       | 36.750       |
| 100 μM puerarin   | 76.759       | 79.807       | 66.230       | 78.271       | 34.470       | 38.995       |
| 50 μM puerarin    | 80.323       | 87.101       | 69.464       | 82.279       | 40.706       | 41.368       |
Table 1. Cytotoxicity of Phytochemicals in Different Cells in Different Exposure Times

| Phytochemical   | 24 h NRU (%) | 24 h MTT (%) | 24 h NRU (%) | 24 h MTT (%) | 24 h NRU (%) | 24 h MTT (%) | 24 h NRU (%) | 24 h MTT (%) |
|-----------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 25 μM puerarin  | 84.751       | 89.632       | 75.674       | 85.421       | 42.758       | 42.447       | 42.758       | 42.447       |
| 10 μM puerarin  | 88.444       | 91.722       | 80.257       | 90.123       | 44.891       | 46.289       | 44.891       | 46.289       |
| 5 μM puerarin   | 91.163       | 97.930       | 87.142       | 99.554       | 48.446       | 48.856       | 48.446       | 48.856       |
| 2 μM puerarin   | 96.215       | 98.212       | 99.285       | 97.664       | 51.635       | 52.698       | 51.635       | 52.698       |
| 1000 μM ursolic acid | 54.032     | 61.724       | 53.392       | 61.457       | 27.483       | 34.463       | 27.483       | 34.463       |
| 800 μM ursolic acid | 54.143    | 70.263       | 56.984       | 62.659       | 28.031       | 35.607       | 28.031       | 35.607       |
| 400 μM ursolic acid | 52.958     | 70.540       | 60.317       | 73.896       | 28.639       | 40.527       | 28.639       | 40.527       |
| 200 μM ursolic acid | 62.704     | 75.929       | 67.797       | 82.222       | 34.348       | 46.396       | 34.348       | 46.396       |
| 100 μM ursolic acid | 70.825     | 78.549       | 70.615       | 83.284       | 35.445       | 49.288       | 35.445       | 49.288       |
| 50 μM ursolic acid | 73.764     | 82.182       | 76.190       | 86.534       | 37.314       | 54.834       | 37.314       | 54.834       |
| 25 μM ursolic acid | 79.809     | 87.348       | 77.718       | 88.743       | 41.765       | 55.028       | 41.765       | 55.028       |
| 10 μM ursolic acid | 82.179     | 94.796       | 79.781       | 91.589       | 44.972       | 53.949       | 44.972       | 53.949       |
| 5 μM ursolic acid | 90.685     | 97.046       | 90.000       | 97.643       | 47.328       | 55.309       | 47.328       | 55.309       |
| 2 μM ursolic acid | 94.543     | 99.417       | 98.075       | 99.405       | 50.741       | 60.898       | 50.741       | 60.898       |

NRU: Neutral red uptake assay, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Table 2. Viability (%) of HeLa cells exposed to galangin, curcumin, pycnogenol, puerarin and ursolic acid

| Phytochemical   | 18 h NRU (%) | 18 h MTT (%) | 24 h NRU (%) | 24 h MTT (%) | 48 h NRU (%) | 48 h MTT (%) |
|-----------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Negative control | 100.000      | 100.000      | 100.000      | 100.000      | 100.000      | 100.000      |
| 1000 μM galangin | 58.388       | 66.607       | 56.372       | 63.003       | 28.426       | 31.157       |
| 800 μM galangin  | 67.179       | 71.258       | 59.919       | 65.681       | 33.411       | 31.117       |
| 400 μM galangin  | 71.308       | 70.253       | 71.913       | 66.483       | 36.102       | 33.633       |
| 200 μM galangin  | 73.759       | 79.332       | 80.607       | 80.417       | 40.276       | 36.840       |
| 100 μM galangin  | 77.114       | 86.896       | 84.335       | 84.080       | 43.396       | 41.520       |
| 50 μM galangin   | 76.319       | 86.006       | 87.156       | 82.111       | 44.670       | 41.832       |
| 25 μM galangin   | 80.393       | 96.149       | 90.845       | 95.513       | 47.389       | 48.074       |
| 10 μM galangin   | 86.929       | 101.936      | 93.649       | 91.786       | 46.417       | 51.415       |
| 5 μM galangin    | 92.023       | 95.899       | 96.603       | 98.088       | 50.841       | 49.454       |
| 2 μM galangin    | 99.959       | 98.790       | 98.343       | 96.028       | 57.574       | 50.343       |
| 1000 μM curcumin | 71.208       | 68.823       | 70.663       | 68.510       | 38.498       | 33.537       |
| 800 μM curcumin  | 69.821       | 76.277       | 71.680       | 75.226       | 42.232       | 33.942       |
| 400 μM curcumin  | 72.047       | 83.150       | 71.555       | 80.417       | 41.973       | 35.971       |
| 200 μM curcumin  | 76.251       | 84.454       | 80.240       | 83.619       | 43.764       | 41.342       |
| 100 μM curcumin  | 76.954       | 92.018       | 84.750       | 91.786       | 46.957       | 43.303       |
| 50 μM curcumin   | 79.184       | 95.899       | 96.603       | 98.088       | 50.841       | 49.454       |
| 25 μM curcumin   | 87.181       | 92.153       | 88.853       | 91.797       | 50.540       | 47.782       |
| 10 μM curcumin   | 89.417       | 98.279       | 89.146       | 97.770       | 53.518       | 48.094       |
| 5 μM curcumin    | 92.465       | 103.645      | 91.759       | 100.508      | 54.381       | 49.164       |
| Table 2. Continue                                                                 |
|----------------------------------------------------------------------------------|
| 2 μM curcumin                                                                   |
| 1000 μM pycnogenol                                                               |
| 800 μM pycnogenol                                                                |
| 400 μM pycnogenol                                                                |
| 200 μM pycnogenol                                                                |
| 100 μM pycnogenol                                                                |
| 50 μM pycnogenol                                                                 |
| 25 μM pycnogenol                                                                 |
| 10 μM pycnogenol                                                                 |
| 5 μM pycnogenol                                                                  |
| 2 μM pycnogenol                                                                  |
| 1000 μM puerarin                                                                 |
| 800 μM puerarin                                                                  |
| 400 μM puerarin                                                                  |
| 200 μM puerarin                                                                  |
| 100 μM puerarin                                                                  |
| 50 μM puerarin                                                                   |
| 25 μM puerarin                                                                   |
| 10 μM puerarin                                                                   |
| 5 μM puerarin                                                                    |
| 2 μM puerarin                                                                    |
| 1000 μM ursolic acid                                                             |
| 800 μM ursolic acid                                                              |
| 400 μM ursolic acid                                                              |
| 200 μM ursolic acid                                                              |
| 100 μM ursolic acid                                                              |
| 50 μM ursolic acid                                                               |
| 25 μM ursolic acid                                                               |
| 10 μM ursolic acid                                                               |
| 5 μM ursolic acid                                                                |
| 2 μM ursolic acid                                                                |
|                                                                                   |
| NRU: Neutral red uptake assay, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide |
|                  | 18 h NRU (%) | 18 h MTT (%) | 24 h NRU (%) | 24 h MTT (%) | 48 h NRU (%) | 48 h MTT (%) |
|------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| **Negative control** | 100.000 | 100.000 | 100.000 | 100.000 | 100.000 | 100.000 |
| 1000 μM galangin | 59.776 | 60.333 | 58.272 | 58.989 | 28.589 | 31.454 |
| 800 μM galangin | 61.264 | 62.857 | 62.119 | 63.301 | 31.015 | 32.675 |
| 400 μM galangin | 65.408 | 64.506 | 66.390 | 64.763 | 33.596 | 34.328 |
| 200 μM galangin | 68.512 | 67.449 | 67.759 | 66.337 | 35.500 | 35.949 |
| 100 μM galangin | 70.992 | 79.984 | 71.915 | 77.078 | 36.271 | 42.579 |
| 50 μM galangin | 75.184 | 87.068 | 76.121 | 84.103 | 37.640 | 46.423 |
| 25 μM galangin | 82.016 | 94.622 | 84.026 | 91.434 | 37.514 | 50.948 |
| 10 μM galangin | 83.872 | 98.075 | 89.900 | 99.630 | 39.725 | 51.458 |
| 5 μM galangin | 97.472 | 98.159 | 96.675 | 99.958 | 47.191 | 52.227 |
| 2 μM galangin | 98.784 | 98.915 | 98.142 | 100.325 | 54.603 | 53.811 |
| 1000 μM curcumin | 59.824 | 60.786 | 57.164 | 60.100 | 32.415 | 33.252 |
| 800 μM curcumin | 62.490 | 61.786 | 59.511 | 61.181 | 32.179 | 34.705 |
| 400 μM curcumin | 64.496 | 66.611 | 60.831 | 66.434 | 34.209 | 36.809 |
| 200 μM curcumin | 74.351 | 76.456 | 70.905 | 79.126 | 34.949 | 36.725 |
| 100 μM curcumin | 88.653 | 87.527 | 87.074 | 86.588 | 35.767 | 42.567 |
| 50 μM curcumin | 92.603 | 94.293 | 91.263 | 93.445 | 41.342 | 44.433 |
| 25 μM curcumin | 92.960 | 95.013 | 92.616 | 94.414 | 44.516 | 45.530 |
| 10 μM curcumin | 91.536 | 97.031 | 93.017 | 98.698 | 45.460 | 49.585 |
| 5 μM curcumin | 92.544 | 97.831 | 92.871 | 99.296 | 46.814 | 50.497 |
| 2 μM curcumin | 97.648 | 99.064 | 97.702 | 99.930 | 47.939 | 50.923 |
| 1000 μM pycnogenol | 35.200 | 39.491 | 34.349 | 33.512 | 17.120 | 16.513 |
| 800 μM pycnogenol | 38.042 | 43.412 | 36.720 | 37.304 | 17.589 | 18.185 |
| 400 μM pycnogenol | 39.680 | 46.636 | 40.456 | 40.307 | 19.544 | 20.785 |
| 200 μM pycnogenol | 41.920 | 48.808 | 42.282 | 50.040 | 21.526 | 25.140 |
| 100 μM pycnogenol | 44.944 | 56.079 | 45.933 | 56.811 | 24.312 | 29.349 |
| 50 μM pycnogenol | 51.088 | 58.468 | 49.845 | 58.898 | 25.193 | 30.355 |
| 25 μM pycnogenol | 53.408 | 66.865 | 56.007 | 67.550 | 25.854 | 35.147 |
| 10 μM pycnogenol | 70.544 | 84.243 | 67.710 | 81.613 | 35.484 | 43.310 |
| 5 μM pycnogenol | 91.888 | 87.303 | 88.443 | 90.637 | 40.897 | 44.223 |
| 2 μM pycnogenol | 98.240 | 97.328 | 96.952 | 96.720 | 42.014 | 45.532 |
| 1000 μM puerarin | 52.384 | 52.263 | 51.964 | 48.410 | 25.885 | 25.584 |
| 800 μM puerarin | 56.882 | 57.004 | 55.175 | 50.662 | 26.467 | 24.906 |
| 400 μM puerarin | 58.336 | 59.103 | 62.184 | 53.354 | 29.064 | 25.390 |
| 200 μM puerarin | 62.653 | 63.222 | 62.642 | 54.300 | 32.305 | 29.887 |
| 100 μM puerarin | 73.376 | 66.454 | 72.290 | 57.206 | 34.729 | 31.926 |
| 50 μM puerarin | 76.768 | 80.315 | 75.240 | 65.467 | 35.374 | 32.109 |

NRU: Neutral red uptake assay, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
CONCLUSION

In conclusion, in this study, the cytotoxic effects of galangin, curcumin, PYC, puerarin and ursolic acid were examined in different cell lines by NRU and MTT assays in 18, 24 and 48 h periods. All of the studied phenolics were decreased the cell viability of both cells with increasing dose. But the cytotoxic effects of phenolics were found more in 48 h incubation period. There is no difference between the results from NRU and MTT assays. 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 confirm beneficial and toxic effects of phenolics.

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