Cytotoxic Effects of Verbascoside on MCF-7 and MDA-MB-231

Verbaskositin MCF-7 ve MDA-MB-231 Üzerindeki Sitotoksik Etkileri

Hülya ŞENOL1*, Pınar TULAY2,3, Mahmut Çerkez ERGÖREN2,3, Azmi HANOĞLU4, İhsan ÇALIS4, Gamze MOCAN5

1Near East University Faculty of Medicine, Department of Medical Biology, Nicosia, North Cyprus
2Near East University Faculty of Medicine, Department of Medical Genetics, Nicosia, North Cyprus
3Near East University, Desam Research Institute, Nicosia, Cyprus
4Near East University Faculty of Pharmacy, Department of Pharmacognosy, Nicosia, North Cyprus
5Near East University Faculty of Medicine, Department of Medical Pathology, Nicosia, North Cyprus

ABSTRACT

Objectives: Verbascoside, also known as acteoside/kusaginin, has attracted a great attention due to its pharmacological features. In this study, we aimed to determine the cytotoxic effects of pure verbascoside isolated from Phlomis nissolii L. plant in both MCF-7 and MDA-MB-231 cell lines in vitro.

Materials and Methods: MCF-7 and MDA-MB-231 cells were treated with verbascoside (100, 48, 25, 10, 1, 0.5, and 0.1 μM) for 24, 48, and 72 hours. Cytotoxic effect of verbascoside in MCF-7 and MDA-MB-231 cells was assessed using TEBU-BIO cell counting kit 8.

Results and Conclusion: IC_{50} values for 24, 48, and 72 h verbascoside exposure of MCF-7 cells were determined as 0.127, 0.2174, and 0.2828 μM, respectively. R^2 values were calculated as 0.9630, 0.8789 and 0.8752, respectively. Two-Way ANOVA multiple comparison test results showed that 100 μM verbascoside has the highest cytotoxic effect on MCF-7 breast cancer (BC) cells after 72 h of exposure. IC_{50} values for 24, 48 and 72 h verbascoside exposure of MDA-MB-231 cells were determined as 0.1597, 0.2584 and 0.2563 μM, respectively and R^2 values were calculated as 0.8438, 0.5107 and 0.9203, respectively. Two-Way ANOVA multiple comparisons test results showed that 100 μM verbascoside has the highest cytotoxic effect on MDA-MB 231 BC cells after 24, 48 and 72 h of exposure.

Key words: Cytotoxicity, MCF-7, MDA-MB-231, Phlomis nissolii L., verbascoside

ÖZ


Gereç ve Yöntemler: MCF-7 ve MDA-MB 231 hücreleri, 24, 48 ve 72 saat süreyle 100, 48, 25, 10, 1, 0.5 ve 0.1 μM verbaskosit ile muamele edildi. Verbsoside MCF-7 ve MDA-MB-231 hücrelerinde sitotoksit etkisi TEBU-BIO hücre sayım kiti 8 kullanılarak değerlendirildi.

Bulgular ve Sonuç: MCF-7 hücrelerinin 24, 48 ve 72 saatlik verbaskoside maruziyetine ilişkin IC_{50} değerleri sırasıyla 0,127, 0,2174 ve 0,2828 μM olarak belirlendi. R^2 değerleri sırasıyla 0,9630, 0,8789 ve 0,8752 olarak hesaplanmıştır. İki yönlü ANOVA çoklu karşılaştırma test sonucu, 100 μM verbaskoside MCF-7 BC hücrelerinde en yüksek sitotoksik etkiye sahip olduğunu gösterdi. MDA-MB 231 hücrelerinin 24, 48 ve 72 saatlik verbaskoside maruziyetine ilişkin IC_{50} değerleri sırasıyla 0,1597, 0,2584 ve 0,2563 μM olarak belirlendi. R^2 değerleri sırasıyla 0,8438, 0,5107 ve 0,9203 olarak hesaplandı. İki yönlü ANOVA çoklu karşılaştırma test sonucu, 100 μM verbaskositin 24, 48 ve 72 saatlik maruziyettinin MDA-MB-231 BC hücrelerinde en yüksek sitotoksik etkiye sahip olduğunu gösterdi.

Anahtar kelimeler: MCF-7, MDA-MB-231, Phlomis nissolii L., verbaskosit
INTRODUCTION

Breast cancer (BC) is the most frequent cancer type found among women, affecting 2.1 million women each year. In 2018, the female deaths due to BC was 627, which compromises 15% of all cancer deaths among women. Furthermore, BC rates in women are higher in more developed regions than in developing countries, and threateningly, these rates are still increasing in every region globally. According to statistical data obtained by the Ministry of Health in North Cyprus, a total of 1.854 men and 1.809 women were diagnosed with cancer between 2012 and 2016. BC has the highest incidence (62.2%) among women in North Cyprus, and this value is lower than the incidence in Europe but unfortunately, however, higher than the BC incidence globally. The current treatment strategies for BC include radiotherapy + adjuvant chemotherapy, radiation therapy, hormone therapy, and surgery have side effects. These may include rib fracture, second non-breast infield malignancies, tissue necrosis, brachial plexopathy in radiation therapy, reduced number of white and red blood cells, elevated risk of infection, anemia, diarrhea, fatigue, hair loss, sore throat, ulcers, nausea, constipation, loss of appetite, and change in skin color during chemotherapy. Due to these side effects, there has been a growing interest in alternative treatment modalities with reduced side effects. There are many studies that have identified anti-cancer properties of herbal medicines that are used in developing countries for medical treatment for many years.

Verbascoside (C₁₅H₂₉O₄), known as acteoside/kusaginin, is a phenylethanoid glycoside. Verbascoside has been isolated from many different plant species such as: Verbascum sinuatum L., Syringa vulgaris, Orobanche raper-genistae, Clerodendron trichotomum Thunb, Phlomis nissolii L. (Lamiaceae), Buddleja brasiliensis, Striga asiatica, Olea europea, Paulownia tomentosa var. tomentosa, Lippia javanica, Lantana camara, and Lippia citriodora. In addition, verbascoside is abundant in olive mill wastewater. There are total of 34 genus Phlomis species found in Turkey and Aegian islands. The project performed on the 33 Phlomis species recorded in the Flora of Turkey resulted in the isolation and characterization of 33 phenylethanoid glycosides, of which verbascoside and forsythoside B were the common compounds for all of the Phlomis species. Recently, two compounds were isolated from the two endemic Phlomis species, P. brevibracteata, and P. cypria growing in Cyprus. Verbascoside attracted great attention due to its pharmacological features such as anti-inflammatory, antioxidant, antimicrobial, antitumor, antitumoral, antimitotic, and cytotoxic effects on many types of cancer such as myeloma and leukemia, human gastric carcinoma, colorectal cancer, human oral squamous cell carcinoma, glioblastoma, and inhibitory effect on tumor cell proliferation. In this study, we aimed to determine the cytotoxic effects of pure verbascoside isolated from the Phlomis nissolii L. plant in both MCF-7 and MDA-MB-231 cell lines in vitro.

MATERIALS AND METHODS

Cell culture conditions

The compound verbascoside used in this study was provided from the studies performed on Phlomis species L. Çalış et al. Human BC cells MCF-7 and MDA-MB-231 (ATCC) were cultured in DMEM/F-12 media supplemented with 10% fetal bovine serum, human insulin of 4 mg/mL, penicillin streptomycin (1%) at 37°C, in a 5% CO₂ containing humidified chamber. The medium was refreshed every other day.

Cell viability/cytotoxicity

MCF-7 and MDA-MB 231 BC cells were plated in 96-well plates in triplicate with a density of 5000 cells/well. The cells were treated with verbascoside after 24 h of culturing at a different concentrations (100, 48, 25, 10, 1, 0.5, and 0.1 μM) for 24, 48, and 72 h. CCK-8 (Tebu, France) analysis was performed according to the manufacturer’s protocol. The absorbencies were measured using Versa max tunable microplate reader at 450nm wavelength.

Statistical analysis

GraphPad Prism software version 8 was used to calculate IC₅₀ values by applying a non-linear regression curve fit analysis. Further, statistical analysis was performed using Two-Way ANOVA multiple comparisons test to determine the significance of a mean difference between the control and varying concentrations of verbascoside.

RESULTS

Cytotoxic effects of verbascoside in MCF-7 cells

To assess the cytotoxicity of verbascoside, MCF-7 BC cells were treated with several concentrations of verbascoside (100, 48, 25, 10, 1, 0.5, and 0.1 μM) for 24, 48, and 72 h. IC₅₀ values of verbascoside in MCF-7 cells are shown in Table 1.

Significance of an mean difference between control and other concentrations of verbascoside for MCF-7 cell line after 24, 48, and 72 h was determined using Two-Way ANOVA multiple comparisons test, and the results are shown in Figure 1-3, respectively.

Two-Way ANOVA multiple comparisons test results for MCF-7 cell line after 24 h exposure to different concentrations of verbascoside showed that the mean difference was not significant at the 95% confidence level (CI) between the control and the test group at 48, 25, and 10 μM verbascoside concentrations. However, significance (at p<0.05) was observed at 100, 1, 0.5, and 0.1 μM verbascoside concentrations, and the control group after 24 h exposure. When concentration of verbascoside was decreased from 100-10 μM, absorbency...
increased so that the number of alive cells increased. When the concentration of verbascoside was further decreased from 10–0.1 μM, absorbency decreased so that number of alive cells decreased but number of dead cells increased. All absorbency values were higher than the control group, indicating that 100, 48, 25, 10, 1, 0.5, and 0.1 μM concentrations of verbascoside were not effectively toxic to the MCF-7 BC cells after 24h exposure (Figure 1).

The mean difference was not significant at 95% CI between the control absorbency value and absorbency values obtained at 100, 48, 25, 10, 1, 0.5, and 0.1 μM verbascoside concentrations after 48 h exposure of MCF-7 cell line. When the concentration of verbascoside was decreased from 100-10 μM, absorbency increased so that the number of alive cells increased. When the concentration of verbascoside was further decreased from 10–0.1 μM, absorbency decreased so that number of alive cells decreased, but the number of dead cells increased. All absorbency values were higher than the control so that 100, 48, 25, 10, 1, 0.5, and 0.1 μM concentrations of verbascoside were not effective on MCF-7 BC cells after 48 h exposure (Figure 2).

The mean difference calculated by Two-Way ANOVA multiple comparisons test was not significant at the 95% CI between the control absorbency value and absorbency values obtained at 100, 48, 25, 10, 1, 0.5, and 0.1 μM verbascoside concentrations after 72 h exposure of MCF-7 cell line. When the concentration of verbascoside decreased from 100-25 μM, absorbency increased so that number of alive cells increased. When the concentration of verbascoside was further decreased from 25–0.1 μM, absorbency decreased so that number of alive cells decreased but number of dead cells increased. The absorbency value at 100 μM verbascoside was the lowest among the other absorbency values, so that lowest number of alive cells but highest number of dead cells was at this concentration. Verbascoside of 100 μM had the highest cytotoxic effect on MCF-7 BC cells after 72 h exposure (Figure 3).

### Cytotoxic effects of verbascoside in MDA-MB 231 cells

MDA-MB 231 BC cells were treated with a several concentrations of verbascoside (100, 48, 25, 10, 1, 0.5, and 0.1 μM) for 24, 48, and 72 h to assess the cytotoxicity of verbascoside by using TEBU-BIO cell counting kit 8. IC$_{50}$ values of verbascoside in MDA-MB 231 cells are shown in Table 2.

Two-Way ANOVA multiple comparisons test results for MDA-MB 231BC cell line after 24, 48, and 72 h are shown in Figure 4–6.

Analysis of the results showed that the mean difference between the control absorbency value and absorbency values increased so that the number of alive cells increased. When the concentration of verbascoside was further decreased from 10–0.1 μM, absorbency decreased so that number of alive cells decreased but number of dead cells increased. All absorbency values were higher than the control group, indicating that 100, 48, 25, 10, 1, 0.5, and 0.1 μM concentrations of verbascoside were not effectively toxic to the MCF-7 BC cells after 24h exposure (Figure 1).

The mean difference was not significant at 95% CI between the control absorbency value and absorbency values obtained at 100, 48, 25, 10, 1, 0.5, and 0.1 μM verbascoside concentrations after 48 h exposure of MCF-7 cell line. When the concentration of verbascoside was decreased from 100-10 μM, absorbency increased so that the number of alive cells increased. When the concentration of verbascoside was further decreased from 10–0.1 μM, absorbency decreased so that number of alive cells decreased, but the number of dead cells increased. All absorbency values were higher than the control so that 100, 48, 25, 10, 1, 0.5, and 0.1 μM concentrations of verbascoside were not effective on MCF-7 BC cells after 48 h exposure (Figure 2).

The mean difference calculated by Two-Way ANOVA multiple comparisons test was not significant at the 95% CI between the control absorbency value and absorbency values obtained at 100, 48, 25, 10, 1, 0.5, and 0.1 μM verbascoside concentrations after 72 h exposure of the MCF-7 cell line. When the concentration of verbascoside decreased from 100-25 μM, absorbency increased so that number of alive cells increased. When the concentration of verbascoside was further decreased from 25–0.1 μM, absorbency decreased so that number of alive cells decreased but number of dead cells increased. The absorbency value at 100 μM verbascoside was the lowest among the other absorbency values, so that lowest number of alive cells but highest number of dead cells was at this concentration. Verbascoside of 100 μM had the highest cytotoxic effect on MCF-7 BC cells after 72 h exposure (Figure 3).

### Table 2. IC$_{50}$ and R$^2$ values for MDA-MB 231 breast cancer cell line

<table>
<thead>
<tr>
<th>Exposure time to verbascoside</th>
<th>IC$_{50}$ (μM)</th>
<th>R$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>0.1597</td>
<td>0.8438</td>
</tr>
<tr>
<td>48 h</td>
<td>0.2584</td>
<td>0.5107</td>
</tr>
<tr>
<td>72 h</td>
<td>0.2563</td>
<td>0.9203</td>
</tr>
</tbody>
</table>
obtained at 100, 48, 25, 10, 1, 0.5, and 0.1 μM verbascoside concentrations was not significant at 95% CI. Absorbency increased when concentration of verbascoside decreased from 100-0.5 μM. This result showed that the number of alive cells increased. A further decrease of concentration of verbascoside from 0.5-0.1 μM caused a decrease of absorbency indicating the decreased number of number of alive cells, but the number of dead cells increased. Absorbency value at 100 μM verbascoside was the lowest among the other absorbency values. This result indicated that lowest number of alive cells, but the highest number of dead cells were at this concentration. Verbascoside of 100 μM had the highest cytotoxic effect on MDA-MB 231 BC cells after 24 h exposure (Figure 4).

The mean difference was not significant at 95% CI between control the absorbency value and absorbency values obtained at 100, 48, 25, 10, 0.5, 1, and 0.1 μM verbascoside concentrations. When concentration of verbascoside was decreased from 100-25 μM, absorbency increased so that the number of alive cells increased. When the concentration of verbascoside further decreased from 25-0.1 μM, absorbency decreased. This result indicated that the number of alive cells decreased, but the number of dead cells increased. The absorbency value at 100 μM verbascoside was the lowest among the other absorbency values. This result showed that the lowest number of alive cells, but the highest number of dead cells, was at this concentration. Verbascoside 100 μM had the highest cytotoxic effect on MDA-MB 231 BC cells after 48 h exposure (Figure 5).

Although the calculated mean difference between the control absorbency value and absorbency values obtained at 48, 25, 10, and 1 μM verbascoside concentrations was not significant at the 95% CI. The mean difference was significant at 95% CI (*significance at p<0.05) between the control absorbency value and absorbency values obtained at 100, 0.5, and 0.1 μM verbascoside concentrations. Absorbency increased when the concentration of verbascoside was decreased from 100-0.5 μM indicating that the number of alive cells increased. When the concentration of verbascoside was further decreased from 0.5-0.1 μM, absorbency decreased so that the number of alive cells decreased but the number of dead cells increased. The absorbency value at 100 μM verbascoside was the lowest among the other absorbency values so that the lowest number of alive cells, but the highest number of dead cells, was at this concentration. Verbascoside of 100 μM had the highest cytotoxic effect on MDA-MB 231 BC cells after 72 h exposure (Figure 6).

**CONCLUSION AND DISCUSSION**

The prevalence of BC has been rising rapidly in the past decades; however, diagnosis and treatment in the early stages is very important. Despite advances in treatment in the early stage of BC, many women experience recurrence and metastasis. Although treatment strategies are limited, the main focus is on medical therapy. The importance of classical treatment methods in cancer therapy is indisputable. Increasing cancer cases and developing resistance to drugs has urged the need for new diagnostic and treatment approaches. Since the success of traditional treatments is limited, most cancer
patients try complementary medical therapies. There has been a growing interest in alternative treatment modalities. Finding alternative therapies with less or no side effects are essential. In recent years, alternative treatment modalities, such as natural products and anti-cancer drugs, have gained importance in BC therapy. Thus, the main aim of this study was to evaluate the cytotoxic effect of verbascoside isolated from \textit{Phlomis nissolii} L. plant (Lamiaceae) in MCF-7 and MDA-MB 231 BC cell lines in vitro.

$IC_{50}$ values for MCF-7 BC cell line after 24, 48, and 72 h exposure to a different concentration of verbascoside were found as 0.127, 0.2174, and 0.2828 μM, respectively. $R^2$ values for 24, 48, and 72 h exposure to verbascoside were calculated as 0.9630, 0.8789, and 0.8752, respectively. Concentrations of 48, 25, 10, 1, 0.5, and 0.1 μM verbascoside were not toxic on MCF-7 BC cells after 24, 48, and 72 h exposure. Verbascoside of 100 μM had the highest cytotoxic effect on MCF-7 BC cells only after 72 h exposure. In a study, verbascoside was isolated from \textit{Scrophularia subaphylla} L., and researchers examined the effect of 1-1000 μg/mL verbascoside on MCF-7 cells and found $IC_{50}$ value as 0.39 (±0.015) μg/mL after 48 h of incubation.\(^{64}\) In another study, 5,6-dihydroxyxanthirrhide was isolated from \textit{Pseuderanthemum carruthersii} (Seem.) Guill. var. atropurpureum (Bull.) Fosb. (Acanthaceae) leaves with 13 different compounds, including verbascoside and the cytotoxic activities of these chemicals, and acetylcholinesterase inhibition against MCF-7 and HeLa cells at a concentration of 100 μg/mL were analyzed. Isoverbascoside and verbascoside showed fairly weak AChE inhibitory activity but showed cytotoxic activity against MCF-7 cells strongly.\(^{65}\) This result supports the results of our study. In another study, acteoside was isolated from the crude methanolic extract of \textit{Leucas indica} flowers, and a range of concentrations of acteoside (250.00, 125.00, 62.50, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98 μg/mL) was tested on the MCF-7 cell line after 48 h of incubation. Researchers evaluated the \textit{in vitro} cytotoxicity of acteoside on MCF-7 cell by using the (3-(4,5-dimethylthiazol-2-

$\beta$-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. This study tested a higher range of acteoside concentration on MCF-7 cell line than our present study, that used a range of concentrations of 100, 48, 25, 10, 1, 0.5, and 0.1 μM verbascoside (acteoside) and obtained higher values of $IC_{50}$ and $R^2$ as 7.7 and 0.9968 than the current study.\(^{66}\) Researchers also concluded that acteoside isolated from \textit{Leucas indica} flowers extract showed a significant cytotoxic activity on MCF-7 cell line, and results indicated that the antiproliferative effect strengthens with an increase in the concentration of the extract (p.2). Results of another study with verbascoside isolated from the aerial parts of \textit{Plantago lagopus} L. showed that verbascoside had strong cytotoxic activities against MCF-7 cell line, and histological analysis proved the apoptotic cell death of MCF-7 cells after the treatment of 50-100 μg/mL verbascoside.\(^{67}\) In one report, the effect of different concentrations of verbascoside isolated from \textit{V. ovalifolium Donn ex Sims} (Scrophulariaceae) on cell viability of MCF-7 cells was measured using the MTT colorimetric assay after 48 h of incubation. The $IC_{50}$ value for verbascoside was calculated as 58.3 μg/mL, and it was observed that verbascoside decreased viability by 69.6% in MCF-7 cells at 100 μg/mL but did not affect the viability of non-tumor MCF-10A cells (up to 100 μg/mL).\(^{68}\) Acteoside may be effective to prevent MCF-7 BC cells because of its antiestrogenic effect. Acteoside isolated from aerial parts of \textit{Verbascum macrurum} exhibited an ER-mediated significant antiestrogenic activity at a low concentration range 10$^{-7}$-10$^{-5}$M in both the ERα and ERβ assay systems, indicating that acteoside may act as antagonist by itself. Acteoside at low concentration (10$^{-7}$ M) demonstrated a potent inhibitory effect against estradiol (10$^{-8}$ M) mainly via Erα, so that acteoside functions as antagonist for ERα-mediated transcription.\(^{69}\) In contrast, in another study, 12 chemical constituents from \textit{Callicarpa nudiflora} were isolated and their cytotoxicity was evaluated by the MTT assay. The cytotoxicity assay demonstrated that the flavonoids luteoloside, lutedin-4’-O-β-D-glucoside, 6-hydroxyluteolin-7-O-β-glucoside, lutedin-7-O-neohesperidoside, rhoifolin, luteolin-7, and 4’-di-O-glucoside showed monolitic proliferation inhibitory activities against Hela, A549, and MCF-7 cell lines in various concentrations. Compounds 6-hydroxyluteolin-7-O-β-glucoside and rhoifolin and iridoid glycoside nudifloside exhibited higher cytotoxic activities.\(^{70}\)

$IC_{50}$ values for MDA-MB 231 cell line after 24, 48, and 72 h of exposure to different concentrations of verbascoside were found as 0.1597, 0.2584, and 0.2563 μM, respectively. $R^2$ values for 24, 48, and 72 h of exposure to verbascoside were calculated as 0.8438, 0.5107, and 0.9203, respectively. Concentrations of 48, 25, 10, 1, 0.5, and 0.1 μM verbascoside are not toxic on MDA-MB 231 BC cells after 24, 48, and 72 h exposure. Verbascoside 100 μM has the highest cytotoxic effect on MDA-MB 231 BC cells after 24, 48, and 72 h exposure. There are few studies about the cytotoxic effects of verbascoside on MDA-MB 231 BC cell line in the literature. In a study, antiproliferative effect of \textit{Strobilanthus crispus} containing verbascoside on MDA-MB 231 cells was evaluated using MTT assay, and the $IC_{50}$ value of methanolic extract was found as 27.2 μg mL$^{-1}$.\(^{71}\) Another study examined the effect of dry olive mill residue water containing verbascoside and found that dry olive mill residue water inhibited MDA-MB 231 cell growth by EC value of 57.15±1.04 c.$^{72}$ Both of these studies support the idea that plant extracts containing verbascoside have cytotoxic effects on MDA-MB 231 BC cell line; however researchers in these studies examined the cytotoxic effects of the plant extracts containing verbascoside and any other chemicals on MDA-MB 231 cell line, unlike pure verbascoside in our study. This study proved that verbascoside isolated from \textit{Phlomis species} L. has cytotoxic effects on MCF-7 and MDA-MB 231 BC cells. Further studies would be performed to assess the underlying mechanisms for apoptotic induction of verbascoside extracted from \textit{Phlomis species} L. In addition, detailed investigations maybe performed to evaluate the synergic effects of verbascoside isolated from \textit{Phlomis species} L. with other plant extracts used in the BC treatment.
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