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Research Article

The Effects of Nobiletin and Devil's Claw in Hela Cervical Cancer Cell Lines

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Abstract

Cervical cancer is the fourth most common type of cancer diagnosed in women. Devil's Claw (*Harpagophytum procumbens*) is a traditional medicine that has been used for a long time. Nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone) is an important component of polymethoxylated flavones found in citrus peels.

HeLa cells were cultured in EMEM medium. HeLa cells were seeded in 96 well plates. After the incubation, plates were read at 570 nm on ELISA reader. Annexin-V technique, based on detection of translocated phosphatidylserine to the outer side of cellular membrane, was used to test the apoptosis promoting ability of test agents on HeLa cells. The Caspase 3/7 activation abilities of test agents on HeLa cells were analysed with caspase 3/7 detection technique.

Devil's claw, nobiletin and the combination of these 2 agents were found to be cytotoxic on human cervix adenocarcinoma cells, HeLa. The cytotoxic effects of all agents were in dose and time dependent. The highest growth inhibiton was detected at the applied highest dose. IC_{50} values for 24 hours were found to be 51.12, 49.03 and 39.2 μg/mL, respectively. This values for 48 hours of application were 43.23, 45.12 and 30.65 μg/mL for Devil's claw, nobiletin and the combination.

Today, combined therapy has come to the fore in the treatment of cancer patients. Nobiletin and devil's claw may be candidates for inclusion in treatment protocols as well as being used alone.

Keywords: HeLa Cells; Devil's Claw; Nobiletin; Cell Viability; Annexin-V

Introduction

Cervical cancer is the second most common female malignant tumor worldwide that seriously threatens women's health [1]. It is the cause of approximately 600,000 cases and 300,000 deaths worldwide in 2018 [2]. Cervical cancer is the fourth most common type of cancer diagnosed in women and the fourth most common cause of cancer-related death in women. Approximately 85% of cervical cancer deaths worldwide occur in underdeveloped or developing countries. The incidence of death from this type of cancer is 18 times higher in low- and middle-income countries than in rich countries [3].

In vitro cell assays are used to study cells in a controlled environment. One of the many usage areas is pharmaceutical studies. Physicochemical properties such as pH, temperature and pressure can be controlled in in vitro analyses [4,5].

MTT is an important colorimetric analysis method that is frequently used in cell viability and cytotoxicity analyses. It is based on the reduction of the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals. This reduction reaction is carried out by metabolically active cells [6]. These cells contain oxidoreductase enzymes and the reaction

is NADPH dependent. MTT is a preliminary analysis method to assess cytotoxicity. Studies indicate that MTT has some limitations. However, the MTT assay is still widely used [7].

Phosphatidylserine is located in the inner plasma membrane. It is released during apoptosis and passes from the inner layer of the plasma membrane to the outer layer. In the outer layer, it binds with a calcium-binding protein, Annexin V. This mechanism is used for apoptosis detection. Fluorescently labeled Annexin V is used for detection. Caspases are a large family of cysteine proteases. This family is required for the initiation and execution of Apoptosis. The cysteine protease Caspase-3 plays the most important role in the family [8].

Devil's Claw (*Harpagophytum procumbens*) is a traditional medicine that has been used for a long time. The most commonly used diseases are allergic reactions, degenerative joint and rheumatologic diseases. The main compounds in its content are iridoid glycosides. The most frequently detected glycosides are harpagoside, harpagit and procumbide glycosides. Among them, the one mainly responsible for the therapeutic activity is harpagoside. Harpagoside content is used as reference standard [9].

Nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone) is an important component of polymethoxylated flavones found in citrus peels. In recent years, beneficial health effects of nobiletin such as anti-carcinogenic, anti-inflammatory, anti-atherogenic, anti-diabetes and anti-obesity have been demonstrated [10-13].

The aim of this study is to examine the effects of Nobiletin and Devil's claw in HeLa cell culture medium.

Materials and Methods

Chemicals

Human cervix adenocarcinoma HeLa (ATCC® CCL-2™) cell line was purchased from the American Type Culture Collection (Manassas, USA). Devil's claw was purchased from Akcan Kimya (Turkey), fetal bovine serum (FBS), penicillin/streptomycin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT), Nobiletin and Eagle's Minimum Essential Medium (EMEM) were purchased from Sigma-Aldrich (St. Louis, USA). Caspase 3/7 and Annexin-V Kits were from (Merck, Millipore, USA).

Cell culture

HeLa cells were cultured in EMEM medium supplemented with 100 units/mL-100 μg/mL of penicillin–streptomycin and 10% fetal bovine serum in 37°C and 5% CO_2 incubator conditions. Celles were passaged twice a week and flasks with confluency of 85% were used in all tests.

Cytotoxicity assessment

Nobiletin and Devil's claw were diluted in DMSO and further dilutions of the stock solutions were prepared in freshly prepared complete growth medium. HeLa cells were seeded (5×10^3/well) in 96 well plates and incubated for 24 and 48 hours in 37°C and 5% CO_2 with the different concetrations of test agents. After that, MTT (5 mg/mL) agent was added (20 μL/well) and cells were further for 3 hours. After the incubation, liquids part of plates were changed with DMSO (200 μL/well) and plates were read at 570 nm (n = 3) on ELISA reader (HTX Synergy, BioTek, USA). The percentages of viability were calculated from the absorbances and IC_{50} concentrations were determined.

Phosphatidylserin translocation assay

Annexin-V technique, based on detection of translocated phosphatidylserine to the outer side of cellular membrane, was used to test the apoptosis promoting ability of test agents on HeLa cells. Briefly, HeLa cells were seeded in six-well plates ($5x10^5$ cells/well) and treated with IC_{50} values of test agents for 24 hours at standard cell culture conditions. After that, cell were collected by trypsinization and were washed in phosphate buffer (PBS). 100 μL/sample of Annexin-V agent was added to all sample containing tunes and further incubated for 15 minutes at dark at room temperature (Muse® Annexin-V and Dead Cell Assay Kit). After the incubation period treated and untreated HeLa cells were analysed with Muse™ Cell Analyzer (Merck, Millipore, Hayward, California, USA).

Caspase 3/7 activation assay

The Caspase 3/7 activation abilities of test agents on HeLa cells were analysed with caspase 3/7 detection technique. In brief, HeLa cells exposed to IC_{50} values of test agents for 24 hours in six-well plates at a density of 5x105/well. After this period, the cells were harvested by trypsinization and washed in phosphate buffered saline (PBS). To the samples for analyses Caspase 3/7 working solution and 7-ADD solutions were added based on user manual of the manifacturer of caspase 3/7 kit (Merck, Millipore, Hayward,

California, USA). Samples were read on a cell analyzer (Muse TM Cell Analyzer, Merck, Millipore, Hayward, California, USA).

Statistical analysis

For analysis of results One way variance analysis for multiple comparisons of GraphPad Prism 6.0 for Windows was used.

Results

MTT results

Devil's claw, nobiletin and the combination of these 2 agents were found to be cytotoxic on human cervix adenocarcinoma cells, HeLa. The cytotoxic effects of all agents were in dose and time dependent. The highest growth inhibition was detected at the applied highest dose. IC_{50} values for 24 hours were found to be 51.12, 49.03 and 39.2 μg/mL, respectively. This values for 48 hours of application were 43.23, 45.12 and 30.65 μg/mL for Devil's claw, nobiletin and the combination. The combination of Devil's claw and nobiletin found to be the most effective cytotoxic on HeLa cells both for 24 and 48 hours of treatment.

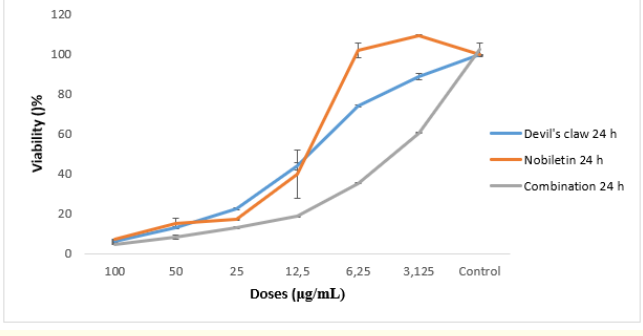

Figure 1: Growth inhibition curves of HeLa cells treated with Devil's claw, Nobiletin and their combination for 24 hours.

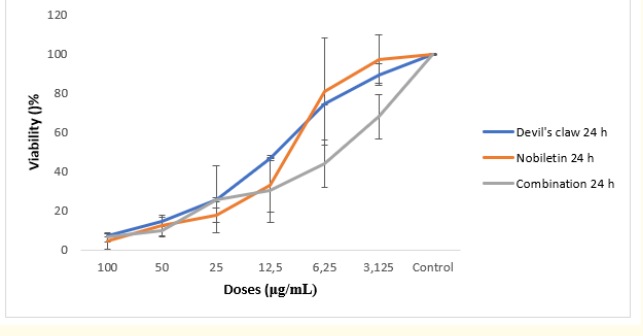

Figure 2: Growth inhibition curves of HeLa cells treated with Devil's claw, Nobiletin and their combination for 48 hours.

Phosphatidylserin translocation assay results

These profiles of HeLa cells showed that tested agents promoted apoptosis (Figure 3). When compared to control cells, agent combination trigered apoptosis in high level on HeLa (Figure 3B). Based on this findings it can be concluded that combination of nobiletin and Devil's claw is proapoptotic on HeLa cells in short time of exposure.

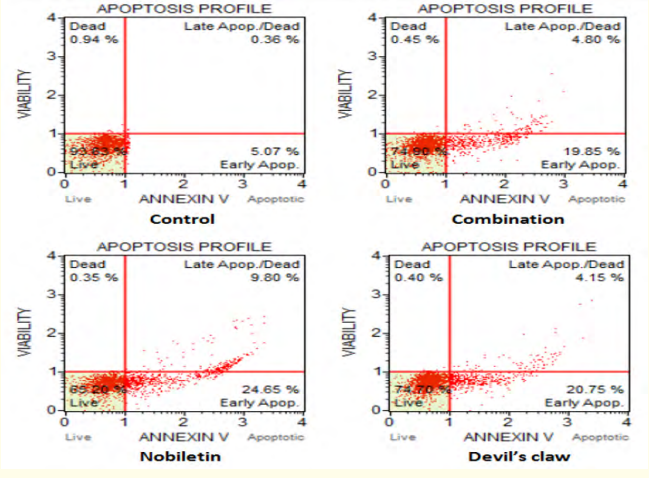

Figure 3: Apoptosis profiles of HeLa cells treated with Devil's claw, nobiletin and the combination for 24 hours. A. Control HeLa cells; Live cells 99.83% were detected. Percentages of late apoptotic cells was 0.36%, early apoptotic cells 5.07% and dead cells were 0.94%. B. HeLa cells exposed to IC50 concentration of agent combination for 24 hours. 74.90% of cells were live. The percentages of dead, early apoptotic and late apoptotic cells were detected to be 0.45%, 19.85% and 4.80%, respectively. C. HeLa cells exposed to nobiletin for 24 hours; Live cells 65.20% were detected. Percentages of late apoptotic cells was 9.80%, early apoptotic cells 24.65% and dead cells were 0.35%. D. HeLa cells exposed to IC50 value of Devil's claw for 24 hours. 74.70% of cells were live. The percentages of dead, early apoptotic and late apoptotic cells were detected to be 0.40%, 20.75% and 4.15%, respectively.

Caspase 3/7 activation findings

The evaluated caspase 3/7 activation profiles of HeLa cells it can be seen that all of the tested agents promoted slightly caspase 3/7 activation that imply to apoptosis (Figure 4). When compared to control cells, all test agents were not detected to triger caspase dependent apoptosis in statistically significant level. Based on the

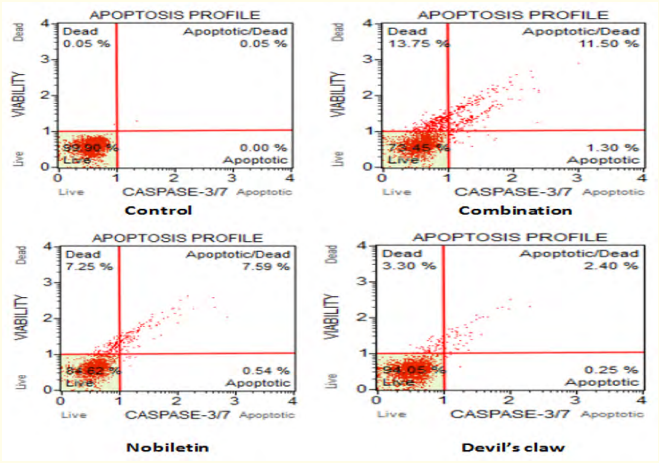

Figure 4: Apoptosis profiles of HeLa cells treated with Devil's claw, nobiletin and the combination for 24 hours. A. Control HeLa cells; Live cells 99.90% were detected. Percentages of apoptotic cells were 0.54% and 7.59% and dead cells were 7.25%. B. HeLa cells exposed to IC50 concentration of agent combination for 24 hours. 73.45% of cells were live. The percentages of dead, and apoptotic cells were detected to be 13.75% and 11.75%, respectively. C. HeLa cells exposed to nobiletin for 24 hours; Live cells 84.62% were detected. Percentages of apoptotic cells were 0.54% and 7.59% and dead cells were 7.25%. D. HeLa cells exposed to IC50 value of Devil's claw for 24 hours. 94.05% of cells were live. The percentages of dead and apoptotic cells were detected to be 3.30%, 2.40% and 0.25%, respectively.

data from caspase 3/7 activation profiles it can be showed that combination of nobiletin and Devil's claw is proapoptotic in the manner of activation of caspases slightly higher then other tested agents on HeLa cells in short application time.

All experimental data of this study together indicated that Nobiletin, Devil's claw and combitanion of that agents were highly cytotoxic on human cervix adenocarcinoma HeLa cells in low doses with antiproliferative and proapoptotic without activation of caspases for short application period of 24 hours. As a conclusion, the combination of nobiletin with Devil's claw worth to be assessed for the further anticancer effects in vitro and in vivo based on the antiproliferative, cytotoxic and proapoptotic activities for short exposure time and at low doses.

Discussion

Devil's claw, nobiletin, and their combination have been determined to be cytotoxic on human cervical adenocarcinoma cells. The effects detected in the analyzes performed were dose- and time-dependent. The greatest growth inhibition occurred when the highest doses were administered. The 24-hour IC_{50} values are 51.12, 49.03 and 39.2 μg/mL, respectively. For 48 hours administration these values are 43.23, 45.12 and 30.65 μg/mL for Devil's claw, nobiletin and combination. The agents tested in our study and their combination support apoptosis. Compared with control cells, the combination in particular induced apoptosis significantly. The obtained caspase 3/7 activation profiles indicate that the agents and their combination promote caspase 3/7 activation. This was not statistically significant for nobiletin and Devil's claw, but was significant for the combination.

A study examined the ability of nobiletin to inhibit the proliferation of SNU-16 cells relative to other flavonoids. Nobiletin induced death of SNU-16 cells via apoptosis. This was demonstrated by increased cell population in the G1 phase, fragmented nuclei, increased Bax/Bcl-2 ratio, increased caspase-3 activity, and proteolytic activation of caspase-9. It was found that the combination of nobiletin and 5-fluorouracil (5-FU) decreased the viability of SNU-16 cells in a concentration-dependent manner, and the combination showed a synergistic anticancer effect. Expression of p53 protein increased with combination therapy. The results of the study suggest that it be combined with nobiletin to increase the efficacy of 5-FU in p53 mutant tumors [14].

Nobiletin has been shown to target P-gp in cancer therapy. A newly published study noted the development of a nobiletin derivative with enhanced solubility and antitumor activity. This agent is known as compound 29d. Compound 29d decreased P-gp activity and increased paclitaxel (PTX) accumulation in lung cancer cells [15,16]. Increasing evidence suggests that nobiletin can induce chemoresistance through the Nrf2/PI3K/Akt and extracellular signal-regulated kinase (ERK) pathways. Compound 29d administration is associated with downregulation of ERK. In addition, nobiletin can inhibit the PI3K/Akt signaling pathway through Nrf2 downregulation [17,18].

Nobiletin and its derivatives target different pathways to sensitize cancer cells to chemotherapy. Another study examined the effectiveness of NOB in increasing the antitumor activity of

PTX. Molecular pathways have been investigated. Nobiletin by downregulation of Nrf2 and Akt and ERK phosphorylation is thought to sensitize lung cancer cells to PTX-mediated apoptosis [18].

Multidrug resistance-associated protein 1 (MRP1), known as ABCC1, was first recognized in lung cancer cells lacking ABCB1 (MDR1 or P-gp) expression [19]. MRP1 inhibition acts as an onco-suppressant and sensitizes cancer cells to chemotherapy [20]. Nobiletin sensitizes lung cancer cells to adriamycin chemotherapy in the same way. Nobiletin increases adriamycin accumulation in cancer cells through downregulation of MRP1, leading to the induction of apoptosis [21].

Studies on the antitumoral efficacy of devil's claw are limited. This indicates the high original value of our study.

The effectiveness of devil's claw has been investigated in numerous studies. These studies range from randomized clinical trials to case reports. Devil's claw primary indication is degenerative joint diseases and low back pain. Harpagoside is the most important compound that contributes to the overall activity of devil's claw. However, it is considered that other compounds in its content may also be effective. However, these effects are not yet fully understood. Studies report that devil's claw preparations should contain daily doses of harpagosides between 30-100 mg [22].

Studies on devil's claw are generally focused on its effects on joint diseases. Findings revealed that treatment with devil's claw improved anxiety and motor coordination disorder in arsenic intoxication. Devil's claw also significantly reduced oxidative stress. It was determined that the activity of devil's claw was correlated with the concentration of harpagoside in serum. This supported that harpagoside is the active ingredient responsible for the neuroprotective effect [23].

Phytochemical research of devil's claw revealed the presence of secondary metabolites. these include glycosides, flavonoids, terpenoids and steroids. These secondary metabolites are further studied to investigate pharmacological effects. The important pharmacological effects of devil's claw are antioxidant and antiangiogenic effects. Disruption of angiogenesis and oxidative stress is the cause of many diseases such as cancer, diabetes and ischemic heart diseases. Therefore, agents that neutralize oxidative stress and the angiogenesis process can be used as modulatory or adjuvant therapy to maintain hemostasis of organisms and reduce disease risk. Disruption of angiogenesis and oxidative stress is also the key to tumoral formations. In this respect, devil's claw can be used for anti-tumoral purposes [24].

A study reported two patients using devil's claw supplements with objective tumor regression confirmed on a computed tomography scan. Both of these patients did not take any herbal or prescription medication other than devil's claw. The role of cox-2 inhibitors and the relationship between devil's claw components and cox -2 are known. Therefore, devil's claw is considered to be worth examining in low-grade lymphoma [25].

Today, combined therapy has come to the fore in the treatment of cancer patients. More than one drug is used in this type of treatment. In addition to increasing the anti-cancer effect with combinations, it is also aimed to prevent drug resistance. Nobiletin and devil's claw may be candidates for inclusion in treatment protocols as well as being used alone.

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