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RESEARCH ARTICLE

Cytotoxic Effect of Some *Digitalis* Species; A Study of Selectivity

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Cytotoxic Effect of Some Digitalis Species; A Study of Selectivity

SUMMARY

Our previous research, on the aerial parts and roots of three different Digitalis species; D.davisiana Heywood, D.viridiflora Lindley and D.grandiflora Miller against HEp-2 and HepG2 cancer cell lines showed that aerial parts were more cytotoxic than roots. So for further research a comparative cytotoxic activity study was performed on the aerial parts of D.davisiana, D.viridiflora and D.grandiflora against HeLa cancer cell line. 3Y1 non-cancerous cell line was used to determine the selectivity. All three extracts showed strong cytotoxic activity on HeLa cells with IC_{50} values less than 15 µg/ml. However, observed cytotoxic activity on 3Y1 cells was found to be lower than that of HeLa cells with IC_{50} values in a range of 94.7-772.3 µg/ml. This finding indicated that different Digitalis extracts showed a selective cytotoxicity against HeLa cancer cell line.

Key Words: *Digitalis species, cytotoxicity, HeLa cell line, 3Y1 cell line, MTT method.*

Bazı Digitalis Türlerinin Sitotoksik Etkisi; Bir Selektivite Çalışması

ÖZET

Daha önceki çalışmamızda üç farklı Digitalis türünün (D.davisiana Heywood, D.viridiflora Lindley ve D.grandiflora Miller) toprak üstü kısımlarının ve köklerinin HEp-2 ve HepG2 kanser hücre serileri üzerinde, toprak üstü kısımların köklere göre daha sitotoksik olduğu gösterilmiştir. Bu nedenle D.davisiana, D.viridiflora ve D.grandiflora'nın toprak üstü kısımların üzerinde gerçekleştirilen araştırma ilerletilerek HeLa kanser hücresi üzerindeki sitotoksik etkisi incelenmiştir. Sağlıklı bir hücre olan 3Y1, selektivitenin değerlendirilebilmesi için kullanılmıştır. Her üç ekstre de HeLa hücreleri üzerinde kuvvetli sitotoksik etki göstermiş ve IC_{50} değerleri 15 µg/ml'nin altında hesaplanmıştır. Ekstrelerin 3Y1 hücreleri üzerinde sitotoksik etkileri daha düşük olmakla birlikte IC_{50} değerleri 94.7-772.3 µg/ml olarak hesaplanmıştır. Elde edilen bu bulgular Digitalis ekstrelerinin HeLa kanser hücresi üzerinde selektif sitotoksik etkili olduğunu göstermektedir.

Anahtar Kelimeler: *Digitalis türleri, sitotoksisite, HeLa hücre serisi, 3Y1 hücre serisi, MTT metodu.*

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INTRODUCTION

In the Flora of Turkey, the genus *Digitalis* (Plantaginaceae) is represented by nine species(Davis, 1978). The genus *Digitalis* has been moved from Scrophulariaceae family to Plantaginaceae family due to the recent chemotaxonomic and phylogenetic studies. *Digitalis* species are known as foxglove. Even there is no report for use of foxglove as a folk medicine, *Digitalis* species have been used in treatment of diseases since 18th century for its cardiac glycosides. The first use of foxglove as a medicine was in 1785 by Withering (Goldman, 2001). Withering observed his patients and showed that as foxglove is a toxic plant, therapeutic index is very close. Foxglove show main effect on heart and kidneys (Baytop, 1999). This effect is due to the increase of sodium levels in cell by the inhibition of Na-K ATPase pump (Katz, 1985). Foxglove also has diuretic effect depending on the dose (Navarro et al., 2000). *Digitalis* species contain biologically active compounds such as cardenolides, phenylethanoid glycosides, flavonoids, and anthraquinones (Ganapaty et al., 2003). Especially, *D.lanata* and *D.purpurea* are very important for their cardioactive glycosides (Mijatovic et al., 2007). Cardiac glycosides have positive inotropic effect so that they are source of important drugs used for the treatment of cardiac insufficiency (Navarro et al., 2000; Fuerstenwerth, 2014). Except these biological activities foxglove also has emetic, antiviral, antibacterial, antifungal, cytotoxic, antiinflammatory and antioxidant activities (Warr et al., 1992; Lopez-Lazaro et al., 2003; Oh et al., 2005; Benli et al., 2009; Orhan et al., 2009; Jin et al., 2011).

Natural products have an important role in cancer therapy. To determine cytotoxic activity is the first step for the new anticancer drug discovery. Cytotoxic activity studies on cardiac glycosides and *Digitalis* extracts show promising results (Zhou et al., 1998; Lopez-Lazaro et al., 2003; Fujino et al., 2015).

Our previous research, on the aerial parts and roots of three different *Digitalis* species; *Digitalis davisiana* Heywood, *D.viridiflora* Lindley and *D.grandiflora* Miller against HEp-2 (Human larynx epidermoid carcinoma) and HepG2 (Human hepatocelular carcinoma) cancer cell lines showed that aerial parts were more cytotoxic than roots (Kutluay et al., 2014). So for further research a comparative cytotoxic activity study was performed on the aerial parts of *D.davisiana, D.viridiflora* and *D.grandiflora* against HeLa (human cervix epithelial cancer cell) cell line. 3Y1 (rat fibroblast cell) cell line was used to determine the selectivity. Use of noncancerous cell line with cancer cell line help to determine selectivity among different cells. Selectivity between different cancer cells and non-cancerous cells helps to point out drug targeting.

MATERIALS AND METHODS

Plant Material

Voucher specimens for all plants were deposited in Hacettepe University Faculty of Pharmacy Herbarium (HUEF). *D.davisiana* Heywood was collected from Antalya, Turkey (HUEF11004) in June, 2011. *D.grandiflora* Miller (HUEF13007) and *D.viridiflora* Lindley (HUEF13008) were collected from Kırklareli, Turkey in July, 2013. *D.davisiana* was identified by Prof. Dr. Hayri Duman (Gazi University Faculty of Science and Literature Department of Biology, Ankara, Turkey), *D.grandiflora* and *D.viridiflora* were identified by Dr. Z. Ceren Arıtuluk (Hacettepe University Faculty of Pharmacy Department of Pharmaceutical Botany, Ankara, Turkey). While *D.davisiana* is endemic to the south coast of Turkey, *D.viridiflora* and *D.grandiflora* are endemic species for Balkans.

Cell Lines

HeLa (human cervix epithelial cancer cell) cell line was provided by Prof. Dr. Nursen Basaran's cell line collection (Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Ankara), 3Y1 (rat fibroblast cell) cell were provided by the Health Science Research Resources Bank (Osaka, Japan).

Chemicals

Fetal bovine serum (FBS), and a minimal essential medium with Earle's salts (MEM-Earle) and antibiotics (penicillin and streptomycin) were obtained from Biochrom AG (Berlin, Germany). Trypsin/EDTA solution was purchased from Merck (Darmstadt, Germany). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was purchased from Sigma (St Louis, USA).

Extraction

The same extraction method was used for all tested plants. Aerial parts of the dried plants powdered and were extracted with methanol four times. Extracts were dried by evaporating under vacuum at 40°C, and were freezedried to remove solvent residue. Then, they were dissolved in water and partitioned with petroleum ether to discard lipophylic fractions. Aquoeus extracts were used for cytotoxic activity tests.

Selective cytotoxic activity was determined through the HeLa cancer cell line and 3Y1 noncancerous cell line by MTT method. Two cell lines were treated with each extract in the concentration range of 1-800 µg/ml.

Cytotoxic Activity

The modified version of Mosmann's MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method was used to determine

cytotoxic activity in our research. HeLa and 3Y1 cell lines were used for cytotoxic activity tests. Cells were seeded in a 96-well plate at a density of $3x10^4$ cells/ml for HeLa cells and $5x10^4$ cells/ml for 3Y1 cells. MEM's Earle medium were used for the culture. Media were supplemented with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were cultured in respective culture media in a humidified 5 % CO_2 in air at 37°C for 24 hours, then were treated with different concentrations of samples for the next 48 hours. After incubation, the cells were washed to discard sample solutions and replaced by 100 µl of fresh medium. 10 µl of MTT solution (5mg/ml in PBS) was added and incubated for 4 hours. After that, 100 µl of 10 % SDS (Sodium dodecyl sulphate) was added to each well to dissolve formazan crystals. The absorbance was measured at 570/620 nm using microplate reader. Results were given as percentage of inhibitory effect treated cells to untreated cells that served as control. Tests were studied as triplicate in this research. Three independent test results were considered, averages and standard deviations were calculated and shown in the figures.

RESULTS AND DISCUSSION

The cytotoxic activity of *D.davisiana*, *D.grandiflora* and *D.viridiflora* extracts tested against HeLa cell line at the concentrations between 1-400 µg/ml. The results showed that all extracts have concentration dependent strong cytotoxic activity against HeLa cell line (Figure 1). For *D.davisiana* and *D.grandiflora* extracts, cell viability is under 50 % even at 5 µg/ml. All the tested extracts showed high cytotoxicity at the concentrations higher than 25 µg/ml. Cell viability is under 10 % at high concentrations (Figure 1).

D.davisiana and *D.viridiflora* extracts caused to increase HeLa cell number when exposed to 1 µg/ml concentration. This result is supported by the findings of Ramirez-Ortega et al. (2006). It was shown that digoxin and digoxigenin isolated from *Digitalis* species cause an increase in HeLa cell number when exposed lower than 10 nM concentration however they also showed that higher concentrations of these compounds have antiproliferative activity (Ramirez-Ortega et al., 2006).

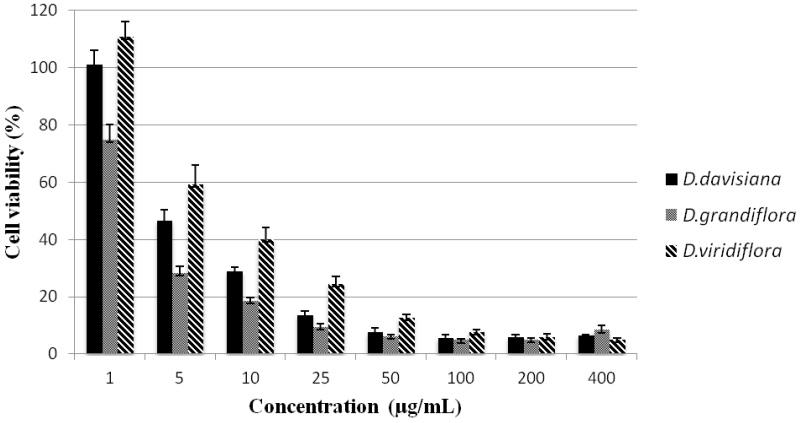

Figure 1. Cytotoxic activity test results against HeLa cell line.

To determine the selectivity of extracts between cancer and non-cancerous cells, 3Y1 cell lines were choosen in this study. Extracts applied at the concentrations between 10-800 µg/ml. Different concentration range was choosen due to the lower cytotoxic activity against 3Y1 cells than HeLa cell lines. We first defined cytostatic activity (cell-growth inhibitory activity) as activity which suppressed the increase in cell number without causing cell death, and cytotoxic activity as which decrease cell number with cell death. These activities were determined by microscopical examination and the dye exclusion method. *D.davisiana* and *D.grandiflora* showed concentration dependent cytostatic activity (cell-growth inhibitory activity) on 3Y1 cell lines. Because, cytotoxic activity of *D.davisiana* and *D.grandiflora* are not significant even at 800 µg/ml (cell viability %, 43.9 and 38.9, respectively). At the concentration of 200 µg/ml, cell viability % of *D.davisiana* and *D.grandiflora* are 45.4 and 48.7 respectively. According to these results, cells were neither dying nor growing due to cytostatic activity. *D.davisiana* was found to be the most active extract among tested *Digitalis* extracts (Figure 2). IC_{50} value for *D.davisiana* is 94.7 µg/ml. *D.viridiflora* extract showed very low cytostatic activity against 3Y1 cells with 772.3 µg/ml IC_{50} value. Even at 400 µg/ml cell viability is more than 80 % with the treatment of *D.viridiflora* (Table 1, Figure 2).

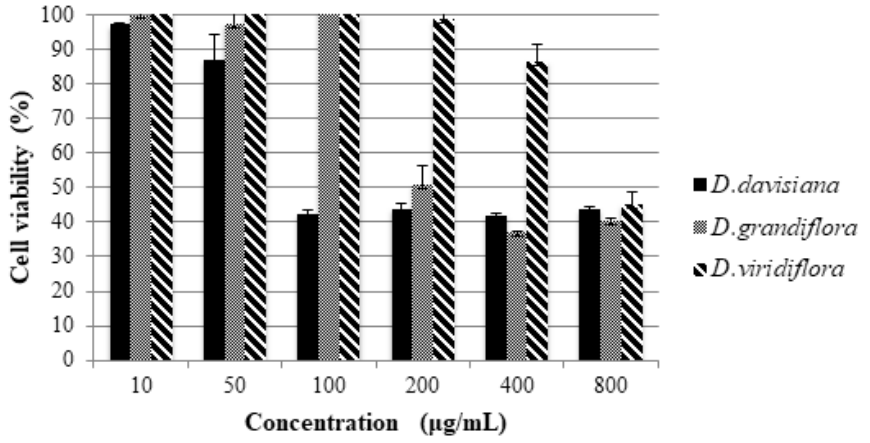

Figure 2. Cytotoxic activity test results against 3Y1 cell line.

All three extracts showed similar strong cytotoxic activity on HeLa cell line with IC_{50} values lower than 15 µg/ml. IC_{50} value for *D.davisiana, D.grandiflora* and *D.viridiflora* is 6.5, 3.8 and 13.2 µg/ml, respectively. However, observed cytotoxic activity on 3Y1 cell line was found to be lower than that of HeLa cell line with IC_{50} values in a range of 94.7-772.3 µg/ml (Table 1). These findings indicated that different *Digitalis* extracts showed a selective cytotoxicity against HeLa cancer cell line.

Table 1. IC_{50} values of extracts on HeLa and 3Y1 cell lines

	IC_{50} values (µg/ml)	
Extracts	**Cell lines**	
	HeLa	3Y1
D.davisiana	6.5	94.7
D.grandiflora	3.8	301.8
D.viridiflora	13.2	772.3

When we compare the present results with our previous research, the tested extracts were found to possess stronger cytotoxic activity on HeLa cell line than HEp-2 and HepG2 cell lines. IC_{50} values of extracts for HEp-2 cell line was between 50.8-79.6 µg/ml and HepG2 was between 211.4-1011.7 µg/ml in our previous study (Kutluay et al., 2014).

In conclusion, according to our cytotoxicity studies on some *Digitalis* species, the extracts showed selective cytotoxicity on the tested cancer and noncancerous cell lines. Moreover, the selectivity was also seen between different cancer cell lines. This selectivity is important for targeting and discovery of new anticancer agents.

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