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RESEARCH ARTICLE

Cytotoxic Potential and Apoptotic Mechanism of Digigrandifloroside: A Cardioactive Glycoside Targeting Caspase 3/7

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SUMMARY

Cardioactive glycosides (CGs) have been used mainly for their positive inotropic effects. However, recent studies on CGs showed their antitumor potential in several cancers. Cytotoxicity of a CG digigrandifloroside was tested against MCF-7 and HeLa cancer cells by the MTT method. Lanatoside A-C, and digitonin were also tested for comparison. To determine the role of apoptosis via caspase 3 and 7, the Caspase-Glo 3/7 test was used. The predicted biological activities of the compounds were also evaluated via the PASSONLINE database. IC50 values for cytotoxic activity of digigrandifloroside against MCF-7 and HeLa cells were found as<100 nM and 252.1 nM, respectively. Digigrandifloroside at 100 nM increased enzyme levels, 1.29 and 2.35 fold, compared to the control cells for MCF-7 and HeLa cells, respectively. These findings suggest the role of apoptosis. Digitonin, a steroidal saponin, showed relatively lower cytotoxicity when compared to CGs on both cells. According to the results obtained from the PASSONLINE database, the potential biological activities of the compounds were reported as, anticarcinogenic activity, antineoplastic activity in lung and breast cancer, caspase 3 and 8 stimulant activity, tp53 expression enhancer activity, together with anti-inflammatory activity, and chemopreventive potential. Digigrandifloroside has an activating effect on caspase 3; this has been proved with the help of biological activity testing. Digigrandifloroside IC50 found for MCF-7 cells is higher than for HeLa cells. It is a well-known fact that MCF-7 cells do not have caspase 3. That is why the higher IC50 found for MCF-7 cells can be connected with the tend to caspase 3 stimulation of digigrandifloroside.

Key Words: *Cardioactive glycosides, Digitalis, Cytotoxicity, MCF-7, HeLa*

Dijigrandiflorozitin Sitotoksik Potansiyeli ve Apoptotik Mekanizması: Kaspaz 3/7'yi Hedefleyen Bir Kardiyoaktif Glikozit

ÖZ

Kardiyoaktif glikozitler (KG'ler) esas olarak pozitif inotropik etkileri için kullanılmıştır. Bununla birlikte, KG'ler üzerinde yapılan son çalışmalar, çeşitli kanserlerde antitümör potansiyellerini göstermiştir. Bir KG olan dijigrandiflorozit'in sitotoksisitesi, MTT yöntemi ile MCF-7 ve HeLa kanser hücrelerine karşı test edilmiştir. Karşılaştırma için lanatozit A-C ve dijitonin de test edilmiştir. Kaspaz 3 ve 7 yoluyla apoptozisin rolünü belirlemek için, Kaspaz-Glo 3/7 testi kullanılmıştır. Bileşiklerin öngörülen biyolojik aktiviteleri de PASSONLINE veritabanı üzerinden değerlendirilmiştir. Dijigrandiflorozit'in MCF-7 ve HeLa hücrelerine karşı sitotoksik aktivitesi için IC50 değerleri sırasıyla <100 nM ve 252,1 nM olarak bulunmuştur. 100 nM'deki dijigrandiflorozit, MCF-7 ve HeLa hücreleri için kontrol hücreleriyle karşılaştırıldığında enzim seviyelerini sırasıyla 1,29 ve 2,35 kat artırmıştır. Bu bulgular apoptozisin rolünü göstermektedir. Steroidal bir saponin olan dijitonin, her iki hücrede de KG'lerle karşılaştırıldığında nispeten daha düşük sitotoksisite göstermiştir. PASSONLINE veritabanından elde edilen sonuçlara göre, bileşiklerin potansiyel biyolojik aktiviteleri antikarsinojenik aktivite, akciğer ve meme kanserinde antineoplastik aktivite, kaspaz 3 ve 8 uyarıcı aktivite, tp53 ekspresyon arttırıcı aktivite, anti-inflamatuar aktivite ve kemopreventif potansiyel olarak bildirilmiştir. Dijigrandiflorozit, kaspaz 3 üzerinde aktive edici bir etkiye sahiptir; bu, biyolojik aktivite testi yardımıyla kanıtlanmıştır. MCF-7 hücreleri için bulunan Digigrandifloroside IC50 değeri, HeLa hücrelerine göre daha yüksektir. MCF-7 hücrelerinin kaspaz 3'e sahip olmadığı iyi bilinen bir gerçektir. Bu nedenle MCF-7 hücreleri için bulunan daha yüksek IC50, dijigrandiflorozitin kaspaz 3'ü uyarma eğilimiyle bağlantılı olabileceği değerlendirilmiştir.

Anahtar Kelimeler: *Kardiyoaktif glikozitler, Digitalis, sitotoksisite, MCF-7, HeLa*

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INTRODUCTION

The genus *Digitalis* has 27 species mainly distributed in northwest Africa, Europe, and Anatolia. They are known as ''foxglove" due to their flower shape named in Latin. (Kreis, 2017). The genus *Digitalis* belongs to the Plantaginaceae family (Albach, Meudt, & Oxelman, 2005). Phenylethanoids, terpenoids, saponins, anthraquinones, flavonoids, and some other compounds have been reported from the genus so far (Kutluay, Ishiuchi, Makino, & Saracoglu, 2019a). *Digitalis* species is well known for its CG content and has been used in the treatment of cardiac diseases for a long while in history (Kreis, 2017).

The first use of cardiac glycosides (CG) as medicine to treat cardiac diseases such as heart failure was reported by William Withering as an extract of *Digitalis purpurea* in 1785 (Prassas & Diamandis, 2008).

In addition to being composed of molecules that are similar in structure, CGs are also a group that exhibits comparable pharmacological effects. Historically, they have been used as arrow poison for hunting and war in Africa, Asia, and South America. Throughout history, it has also been used as an emetic, diuretic, and heart tonic (Morsy, 2017).

These structures are distributed in several genera in some families, such as Apocynaceae (*Adenium*, *Acokanthera*, *Strophanthus*, *Apocynum*, *Cerbera*, *Tanghinia*, *Thevetia*, *Nerium*, *Carissa,* and *Urechites*), Asclepiadaceae (*Gomphocarpus*, *Calotropis*, *Pachycarpus*, *Asclepias*, *Xysmalobium*, *Cryptostegia*, *Menabea,* and *Periploca*), Liliaceae (*Urginea*, *Bowiea*, *Convallaria*, *Ornithogalum,* and *Rohdea*), Ranunculaceae (*Adonis* and *Helleborus*), Moraceae (*Antiaris*, *Antiaropsis*, *Naucleopsis*, *Maquira,* and *Castilla*), Cruciferae (*Erysimum* and *Cheiranthus*), Sterculiaceae (*Mansonia*), Tiliaceae (seeds of *Corchorus*), Celastraceae (*Euonymus*, *Lophopetalum*), Leguminosae (*Coronilla*) and Plantaginaceae (*Digitalis*) (Evans, 2002).

Epidemiological studies reported that people who use CGs as a treatment have a lower rate of death risk of cancer (Prassas & Diamandis, 2008). After the first studies held in the second half of the 20th century, researchers have reported so many findings for the anticancer activity of CGs (Ainembabazi, Zhang, & Turchi, 2023). Among cancer, breast, lung, colorectal, prostate and stomach leads in the number of diagnosed patients (Sung et al., 2021).

CGs have been shown to inhibit Na+/K+-ATPase and induce the immune system to show anticancer activity. CGs block the Na+/K+-ATPase pump, causing elevated levels of intracellular calcium that are sufficient to lead to cell death in cancer cells (Prassas & Diamandis, 2008; Skubník, Pavlícková, & Rimpelová, 2021). Even though Na+/K+-ATPase is accepted as one of the mechanisms of action for CGs the whole process has not been elucidated yet. In a study by Gupta et al., they reported that CGs were 100-fold cytotoxic to human cells when compared with rodent cells. Rodent Na+/K+-ATPase activity was shown to be inhibited in higher concentrations of CGs (Gupta, Chopra, & Stetsko, 1986). In the past few years, many studies have revealed that cardiac glycosides show anticancer activity by blocking different signal transduction pathways implicated in cell proliferation and cell survival. Digoxin, digitoxin, lanatoside C, oleandrin, and some other CGs were studied in detail to decipher the effects and mechanism of action of these compounds (Duan et al., 2021; Durmaz et al., 2016; Karakoyun et al., 2021; Yang et al., 2022).

Digigrandifloroside (13- epidigoxigenin 3-*O*-β- glucopyranosyl (1→4) digitoxopyranoside), is a CG that was isolated from *D. grandiflora* Miller, an endemic plant for Balkan region. Digigrandifloroside was reported to show promising results on the HEp-2 cell line (Kutluay, Makino, Inoue, & Saracoglu, 2019b).

In this study, we aimed to compare the cytotoxicity potential of the compound with other well-known CGs such as lanatosides A-C and a saponin called digitonin which was previously isolated by *Digitalis* species. For this purpose the potential biological ac-

tivities were evaluated and experimentally tested.

MATERIAL AND METHOD

Compounds

Digigrandifloroside was obtained from our previous isolation studies (Kutluay, Makino, et al., 2019). Lanatosides A-C (Fluka, AG, Buchs, Germany) and digitonin (Merck, Germany) were used in biological activity assays. MEM's Earle medium and Fetal bovine serum were obtained from Sigma Aldrich. Penicillin-streptomycin and trypsin (Biowest, France), Caspase 3/7 Glo assay kit (Promega Corporation, Madison, WI, USA), MTT (Sigma Aldrich), dimethylsulfoxide (Merck, Germany) were purchased.

Biological activity prediction

Using the PASSONLINE database (https://www.way2drug.com/PASSOnline/index.php), the biological activities of digigrandifloroside, lanatosides A–C, and digitonin were predicted (Filimonov et al., 2014). This extensive database offers information on the possible actions of substances in over 4,000 biological processes. The Pa (probability to be active) and Pi (probability to be passive) values are used to express the results. If a compound's Pa value is higher than its Pi value, suggesting a higher probability of biological action, it is deemed potentially active.

Cytotoxic activity

For cytotoxic activity assays, HeLa (human cervical carcinoma), and MCF-7 (human breast adenocarcinoma), cell lines were employed. A volume of 100 µL of cells was seeded into a 96-well plate at a density of 3×10^4 cells/mL for HeLa and 8×10^4 cells/mL for MCF-7. MEM's Earle medium was used to culture cells. The cells were maintained in media supplemented with 10% FBS and 1% penicillin-streptomycin solution in a humidified atmosphere containing 5% CO_2 at 37 °C for 24 h. Subsequently, the cells were exposed to various concentrations of samples (0.1-10 µM) for an additional 48 h. Following incubation, the cells were washed and the medium was replaced with fresh media. To each well, 10 µL of MTT solution (5 mg/mL in phosphate-buffered saline) was added and incubated for 4 h. Then, 100 µL of dimethylsulfoxide was added to dissolve the formazan crystals produced by viable cells. Absorbance was measured at 570/620 nm using a microplate reader. The results were presented as the percentage of inhibition in treated cells compared to untreated control cells (Saracoglu, Inoue, Calis, & Ogihara, 1995). The averages of three independent tests were calculated.

Determination of caspase 3/7 activity

HeLa (ATCC: CCL-2) and MCF-7 (ATCC: HTB-22) cell lines were cultured in their respective growth media, which were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and maintained in a humidified incubator at 37°C with 5% CO_2. For the Caspase-Glo 3/7 assay (Promega Corporation, Madison, WI, USA), cells were seeded into 96-well white-walled plates at a density of cells 3×10^4 cells/mL for HeLa and 8×10^4 cells/mL for MCF-7 and allowed to adhere through 24-h incubation. After that test compounds were added to the cells; control wells received an equivalent volume of vehicle. Following the 48 h treatment, the Caspase-Glo Reagent was brought to room temperature, and an equal volume of this reagent was subsequently added into each well. The plates were subsequently incubated in the dark at room temperature and gently shaken using a plate shaker for 30 seconds. We assayed luminescence using a plate-reading luminometer (BioTek Instruments, Winooski, VT, USA), with the signal indicative of caspase-3/7 activity. All luminescence readings of treated wells were divided by the average control well to calculate relative caspase-3/7 activity that was plotted as percent control.

RESULTS and DISCUSSION

Compounds; 4 CG named digigrandifloroside, lanatoside A-C, and a steroidal saponin digitonin were selected for evaluation and comparison of their role in selected cancer cells (Figure 1). One of the compound selection criteria was to compare the aglycone of CGs. Lanatosides A-C has all the same sugar moiety but

different types of aglycones. Lanatoside A has digitoxigenin, Lanatoside B has gitoxigenin, Lanatoside C has digoxigenin skeleton. Digigrandifloroside has epidigoxigenin skeleton. To compare the cytotoxicity profile of CGs with a steroidal saponin which was also isolated from the *Digitalis* genus previously used.

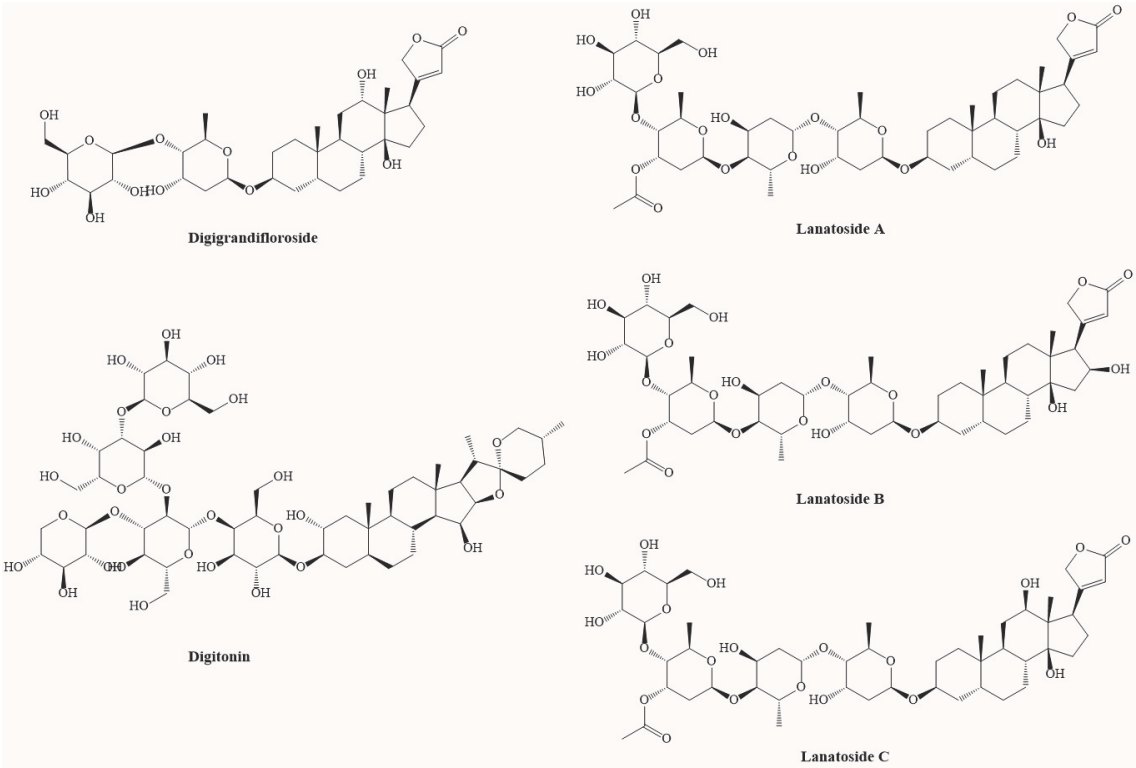

Figure 1. The chemical structures of the compounds tested in the study

Biological activity prediction of selected compounds

To assess the potential targets before *in vitro* and *in vivo* studies, there are some tools used for biological activity prediction. PASS (Prediction of Activity Spectra for Substances) is one of the applications that is used often in research studies. This software provides predictions of various types of biological activities. This biological activity can consist of pharmacotherapeutic effects, biochemical mechanisms, toxicity, metabolism, and gene expression regulation (Filimonov et al., 2014). All selected compounds were applied to the software and results were obtained. The probability of the results is given as the probability of being active 'Pa' and the probability of being inactive 'Pi'. Results with higher values of Pa than 0.7 were selected as a threshold. Predicted biological activities are given in Supplementary Table 1 and Figure 2. Cancer is a complex disease with underlying several mechanisms. The potential biological activities of the compounds were elucidated. Anticarcinogenic activity, antineoplastic activity in lung and breast cancer, caspase 3 and 8 stimulant, tp53 expression enhancer activity, together with anti-inflammatory activity, and chemopreventive potential were reported as potential biological activities of these compounds related to cancer.

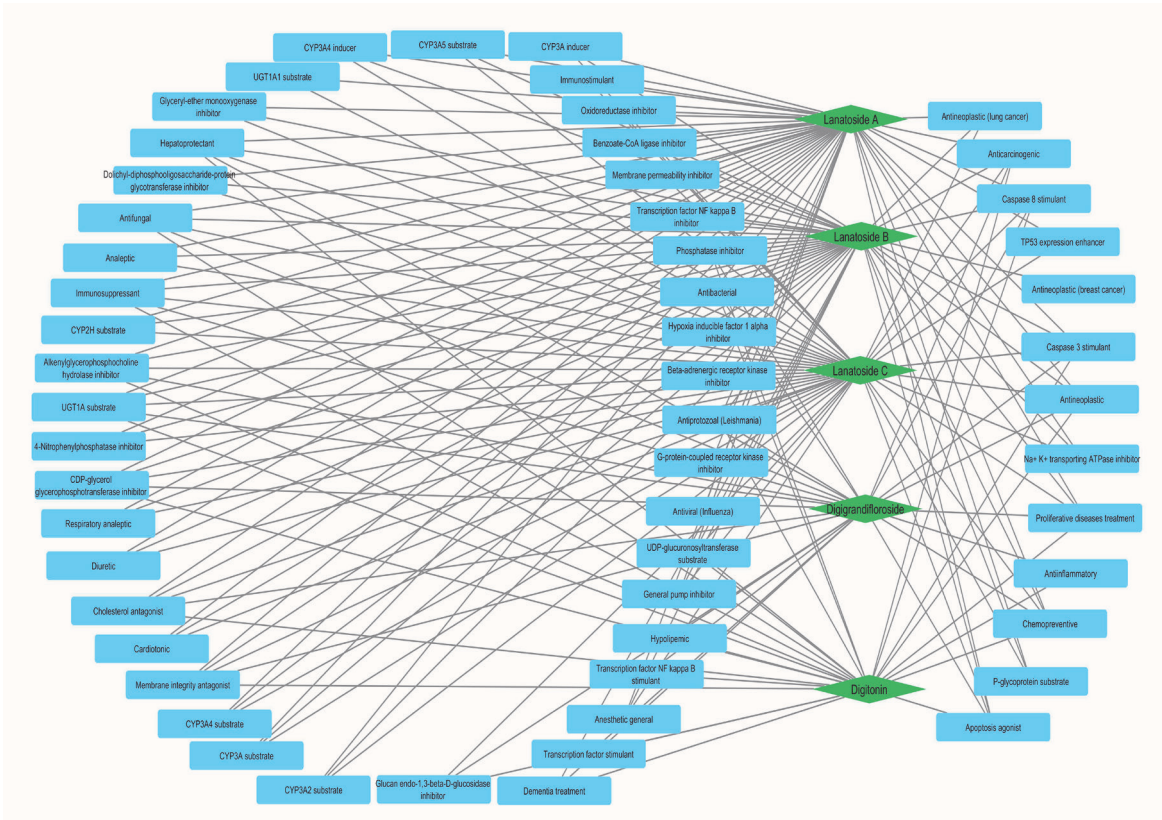

Figure 2. The predicted biological activities of digigrandifloroside, lanatosides A-C, and digitonin. Blue nodes represent the biological activity green nodes represent the compounds.

Previous studies on CGs show their potential for chemopreventive activity. People who use CG as a treatment clinically were reported to have a lower prevalence of death risk from cancer (Prassas & Diamandis, 2008). From these predicted biological activities, breast cancer, and caspase 3 levels were experimentally evaluated in our study. Experimental validation was performed testing the cytotoxicity of compounds on MCF-7 and HeLa cell lines and determination of caspase 3/7 levels.

Cytotoxic activity of CGs and digitonin

To evaluate the cytotoxicity of the compounds MCF-7 and HeLa cancer cell lines were selected. Compounds digigrandifloroside, lanatosides A-C, and digitonin were tested at 0.1-10 µM. CGs showed higher cytotoxicity when compared with digitonin. The IC_{50} value of digitonin against MCF-7 and HeLa cells were found as 1,12 µM and 0,66 µM, respectively (Table 1).

Table 1. IC_{50} values of extract and compounds against HeLa and MCF-7 cell lines

Compounds	Cell lines	
	HeLa	**MCF-7**
Digigrandifloroside	252.1 nM	<100 nM*
Lanatoside A	<100 nM*	<100 nM*
Lanatoside B	<100 nM*	<100 nM*
Lanatoside C	<100 nM*	<100 nM*
Digitonin	662,6 nM	1120.1 nM

* The IC_{50} value was lower than the tested minimum concentration of 100 nM

Tested CGs showed higher cytotoxicity on MCF7 cells, whereas digitonin was more cytotoxic on the HeLa cell line (Figures 3 and 4.). The cell viability was decreased by under 40% in both cells with even 0.1 μM lanatosides A-C application. Digigrandifloroside showed similar cytotoxicity on the MCF-7 cell line but it decreased the cell viability to 60% at 0.1 μM concentration (Figure 4.).

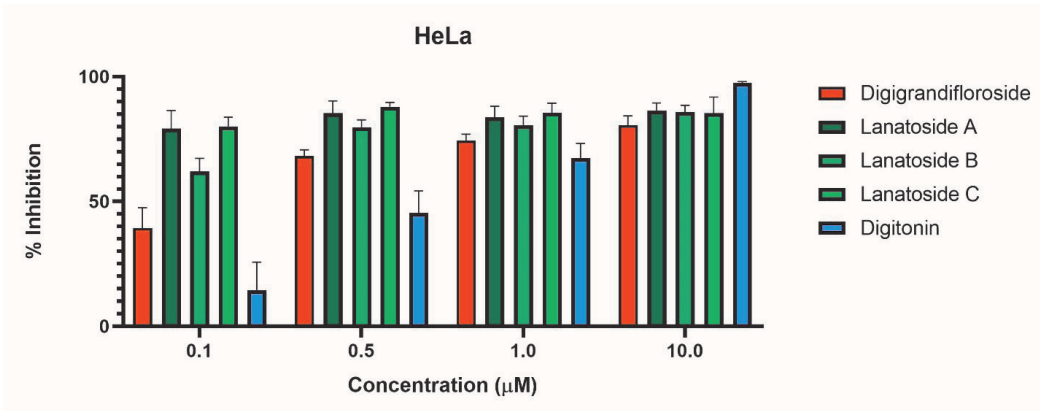

Figure 3. Cytotoxic activity of CGs and digitonin on HeLa cell line using MTT method. Results are given as inhibition %, and expressed as mean ± S.D. (n=3)

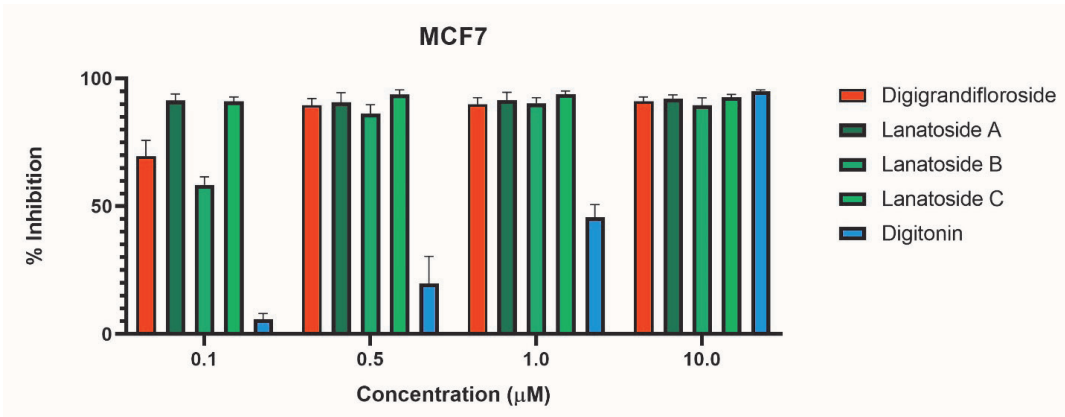

Figure 4. Cytotoxic activity of CGs and digitonin on MCF-7 cell line using MTT method. Results are given as inhibition % and expressed as mean ± S.D. (n=3)

When we compare different aglycon types of CGs via Lanatosides A-C, the results showed that both digitoxigenin and digoxigenin type CGs showed higher cytotoxicity than gitoxigenin. Also, epimerisation from C-13 of digoxigenin might have a role in the decrease in cytotoxic activity as Lanatoside C showed higher cytotoxic activity than digigrandifloroside. But in this comparison, the sugar units of the compounds are also different but the potential bioactivity of CGs depends on their structure of aglycon.

There are several reports of lanatoside C against cancer in the literature (Chao et al., 2017; Duan et al., 2021; Durmaz et al., 2016; Hu et al., 2018; Reddy, Kumavath, Ghosh, & Barh, 2019). In a previous study on lanatoside C, Reddy et al. reported the IC_{50} value as 0.4 μM on the MCF-7 cell line and suggested the potential mechanisms as arresting G2/M phase through blocking MAPK/Wnt/PAM signaling pathways and stimulating apoptosis via inhibition of PI3K/AKT/mTOR signaling pathways (Reddy et al., 2019). In a study on hepatocellular carcinoma lanatoside C induced apoptosis through protein kinase activation (Chao et al., 2017). Lanatoside C was also reported to induce cell cycle arrest at the S and G2/M phases. In the same study lanatoside C was also reported to inhibit JAK-2-STAT6 signaling and induce apoptosis (Duan et al., 2021). Studies on lanatoside C indicate that it can both induce intrinsic or extrinsic pathways of apoptosis and is one of the most studied compound among the group of CGs (Schneider, Cerella, Simoes, & Diederich, 2017). The only study on digigrandifloroside reported the cytotoxicity of the compound on HEp-2 cells. In the same study, it was found that digigrandifloroside showed cytostatic activity on normal cell line L929 where it was cytotoxic on cancer cell line Hep-2 at the same concentrations. The selectivity of the compound between cancer and non-cancerous cells was reported (Kutluay, Makino, et al., 2019).

The other compound tested in our studies was digitonin, a spirostan saponin, reported to have a hemolytic effect and a role in membrane permeabilization (Korchowiec, Janikowska-Sagan, Kwiecinska, Stachowicz-Kusnierz, & Korchowiec, 2021). Digitonin is also reported for its anticancer activity and enhancing other secondary metabolites' cytotoxicity. The effect on the cell membrane causes an increase in cell membrane permeability and helps polar cytotoxic compounds to enter through the cell membrane (Eid, El-Readi, & Wink, 2012).

Determination of caspase 3/7 activity

Caspase 3/7 levels were measured for digigrandifloroside on MCF-7 and HeLa cells. Apoptosis involves the roles of caspases 3 and 7. Caspase 3 regulates apoptosis's morphological alterations and DNA fragmentation. When caspase-3 is present, cell death occurs more effectively. Caspase 7 is involved in both inducing apoptosis and releasing cells from the extracellular matrix. It is well known that MCF-7 cells lack caspase 3 (Laffin, Chavez, & Pine, 2010). Taking into consideration this situation, our results support this. Caspase 3/7 levels increased 1.29-fold in MCF-7 cells and 2.35-fold in HeLa cells. The results showed that digigrandifloroside has increased caspase levels in both cells (Figure 5.). The 1.29-fold increase is upon the induction of caspase 7 but not caspase 3 in MCF-7 cells.

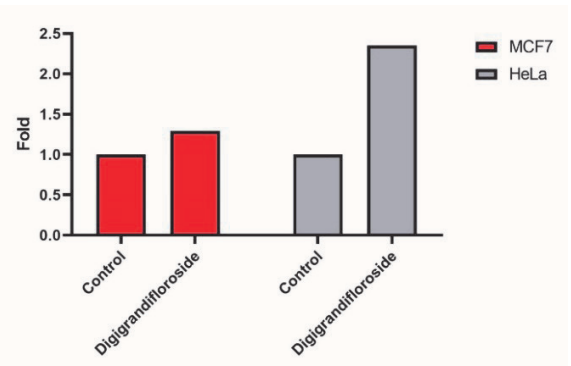

Figure 5. Caspase 3/7 activity of digigrandifloroside in the MCF-7 and HeLa cell lines

CONCLUSION

Overall, the diverse actions of cardiac glycosides in cancer treatment highlight their canonical role in this space and consequently emphasize. Researchers focus

on CGs to determine their role and the mechanisms against several cancers. Na+/K+-ATPase inhibition is one of the major mechanisms in the cytotoxicity of this group of compounds. In addition, they induce cell cycle arrest at different stages, and induce apoptosis via several protein kinases and caspases. In our study an unusual CG, digigrandifloroside, with an aglycone of 13-epidigoxigenin was tested on 2 different cancer cells, and caspase 3/7 levels were evaluated. The study showed that digigrandifloroside might induce apoptosis via both caspase 3 and 7. The biological activity prediction studies performed in our research showed that digigrandifloroside has a caspase 3 stimulatory effect which was validated by *in vitro* assays in our study. The IC_{50} value of digigrandifloroside is found to be higher in MCF-7 cells when compared to HeLa cells. MCF-7 cells are known to be lack of caspase 3. This might explain the higher IC_{50} value and caspase 3 stimulation of digigrandifloroside.

Digoxigenin and digitoxigenin-type CGs demonstrated more cytotoxicity than gitoxigenin when we compared the various aglycon of CGs using Lanatosides A–C. Furthermore, as digoxigenin's C-13 epimerization exhibited a lower cytotoxic activity than digigrandifloroside, this could potentially account for the drop in cytotoxic activity. Although the compounds' sugar units differ, the aglycon structure of CGs determines their potential bioactivity.

These findings might be useful for future studies and mechanisms underlying the cytotoxicity of these compounds should be investigated further.

AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis (VMK), experimenting (VMK), preparing the study text (VMK), reviewing the text (VMK, İS), analysis and interpretation of the data (VMK, İS), literature research (VMK)

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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