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# *IN VITRO* CYTOTOXICITY OF CARDIAC GLYCOSIDE 17βH-NERIIFOLIN ISOLATED FROM *CERBERA ODOLLAM* AGAINST CERVICAL (HeLa), LUNG (A549) AND PROSTATE (DU145) CANCER CELL LINES

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Cardiac glycosides (CGs) are a group of compounds with glycone (sugar) and aglycone (steroid and lactone ring) structures, which can be isolated from certain plants and amphibians. The earliest drugs used to treat heart failure were CGs such as digoxin and digitoxin due to their antiarrhythmic properties. Later, some CGs were also reported to have anticancer properties. In this study, we investigate the anticancer potential of a CG, which is 17βH-neriifolin, isolated from *Cerbera odollam* leaves. This study aimed to evaluate the anticancer properties of 17βH-neriifolin against selected cancer cell lines and compare its anticancer activities with other CGs, namely digitoxin, digoxin and ouabain. The *in vitro* anticancer activities of these compounds were assessed against cervical (HeLa), lung (A549) and prostate (DU145) cancer cell lines using Sulforhodamine B (SRB) assay following 72 hours of treatment. Paclitaxel was used as the control drug. $IC_{50}$ values were determined from concentration-response curves by plotting the percentage of cell viability against concentrations of the compounds. The results revealed that 17βH-neriifolin was found to be the most active in inhibiting the proliferation of cancer cells, with $IC_{50}$ values ranging from 5.3 to 16 nM followed by ouabain (10–35 nM), digitoxin (22–120 nM) and digoxin (27–600 nM) in all cancer cells tested. These findings suggest that 17βH-neriifolin—which targets the sodium potassium ATPase, a promising new anticancer drug target—has a broad spectrum of activity against various cancer cells and warrant further investigation in the drug discovery phase.

Keywords: 17βH-neriifolin, cardiac glycoside, SRB assay, *in vitro* anticancer, cytotoxicity

## INTRODUCTION

Lung and prostate cancers are the top two commonly occurring cancers among men, whereas cervical cancer is the fourth most common cancer in women (WHO 2024). Early detection of cancer can improve the chance of survival. However, cancers detected at a very late stage, where metastasis has occurred, generally have poor prognoses. Chemotherapy is often used to treat metastatic cancer, either as a sole treatment or adjuvant therapy. For example, paclitaxel and cisplatin are still used to treat various metastatic cancers, including lung, prostate and cervical cancers (Gilligan & Kantoff 2002, Liu et al. 2011, Della Corte et al. 2020, Yang 2023). Despite this, survival rates remain poor, often less than 20% in lung cancer patients (Bray et al. 2022), indicating a critical need for new anticancer agents. Natural products, particularly those derived from plants, continue to be a valuable source for developing new chemotherapy drugs (Newman & Cragg 2020). A recent trend in chemotherapy drug development is the search for new candidates with unique or multiple drug targets to overcome drug resistance and minimise toxicity side effects.

Cardiac glycosides (CGs) have a long history since 1785 when they were used to treat cardiovascular disease (Wray 1985). However, there has been a substantial increase in reports highlighting the anticancer potential of CGs isolated from various plant species, as well as existing CG drugs such as digitoxin, digoxin and ouabain, which inhibit sodium-potassium ATPase, a promising new anticancer drug target (Newman et al. 2008, Wang et al. 2021). The inhibition to $Na^+$,$K^+$-ATPase by CGs at the cell membrane is not merely viewed as a simple interruption of ion-exchange pump, but rather

a trigger to a series of complex events within the cytosol, mitochondria and nucleus that lead to apoptosis. They have shown that binding to $Na^+$,$K^+$-ATPase increases the (cell surface) expression of death receptors 4 (DR4) and 5 (DR5) thereby activate caspase activity (Frese et al. 2016); reduces the expression of transcription factors such as hypoxia-inducible factor 1 (HIF-1) (Zhang et al. 2008); inhibits Akt activation that block apoptosis (Pongrakhananon et al. 2013); blocks the transcription factor NF-κB (Yang et al. 2005); inhibits binding of tumor necrosis factor receptor 1 protein-associated death domain (TRADD) to cellular membranes thereby inhibiting tumor necrosis factor (TNF) (Yang et al. 2005); activates the Ras pathway (Valente et al. 2003); inhibits Fas-related signalling (Panayiotidis et al. 2010) and decreases the anti-apoptotic proteins Bcl-XL and Bcl-2 as well as topoisomerase I and II (Winnicka et al. 2008), among others. Although the primary target of CGs is $Na^+$,$K^+$-ATPase, not all reported anticancer drugs inhibit this important enzyme. CGs should be thought as anticancer agents with multiple mechanisms of action. Sources of CGs from the Apocynaceae family include cerberin and tanghinin from Cerbera manghas, odollin from *Cerbera odollam*, oleandrin from *Nerium oleander*, ouabain from *Strophanthus gratus* and thevefolin from *Thevetia peruviana*, among others (Rowe 1916, Wen et al. 2016). Our group discovered a CG, 17βH-neriifolin, from *Cerbera odollam* leaves (Siti Syarifah et al. 2011).

*Cerbera odollam* (Apocynaceae) is found along the shores of the Indian Ocean, through Malaysia, to the Pacific Islands (Burkill 1966). This medium-sized tree can grow up to 12 meters tall and has a rounded, bushy crown. The leaves are glossy green, narrowly obovate to elliptic, with a short drip-tip, as shown in Figure 1. *C. odollam* resembles *C. manghas*, but the two can be distinguished by the colour of their corolla, fruit shape and the colour of the ripe fruit. *C. odollam* features a yellow-eyed white corolla and spherical fruit that turns green when ripe, whereas *C. manghas* has a red-eyed white corolla and oblong fruit that turns reddish when ripe (Cheenpracha et al. 2004). The ethnobotanical uses of *C. odollam* include applying its oil topically to treat itch, as a body rub to treat colds, as an insect repellent for hair, and rubbing the fresh fruit on legs to relieve rheumatism (Burkill 1966). Although the fruit is known to be poisonous due to the presence of cerberin, other parts of the plants have been reported to have cytotoxic properties (Chan et al. 2016). These *in vitro* cytotoxic effects had been reported on 17βH-neriifolin on small cell lung cancer (SCLC), oral epidermoid, breast (Laphookhieo et al. 2004), acute myeloid leukemia (Takase et al. 2022) and colon cancer cells (Chang et al. 2000), but there is limited information available on the studies against non-small cell lung cancer (NSCLC), cervical and prostate cancer cells. Hence, this study investigates whether 17βH-neriifolin can exert better cytotoxic effects *in vitro* compared to other prominent CGs, including digitoxin, digoxin and ouabain.

## MATERIALS AND METHODS

### Compounds

17βH-neriifolin (≥95% pure by HPLC) was isolated from the leaves of *C. odollam* based on the previously described method in Nurhanan Murni et al. (2020). A voucher specimen (AC5832-P) was deposited at the Forest Research Institute Malaysia herbarium. Cytotoxic chemicals such as digitoxin, digoxin, ouabain and paclitaxel were purchased from Sigma Aldrich (St. Louis, MO, USA).

### Cell lines

Cancer cell lines included for this study were cervical (HeLa), lung (A549) and prostate (DU145), while the normal cell line used was liver (WRL-68). All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Stock cultures were grown in T-25 flasks containing 5mL of Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco Life Technologies, Paisley, UK), 1% (v/v) penicillin-streptomycin, 1% (v/v) gentamicin and 1% (v/v) amphotericin B from Capricorn Scientific GmbH, Ebsdorfergrund, Germany; and incubated in 5% carbon dioxide and 37°C. Growth medium was changed at 72-hour intervals.

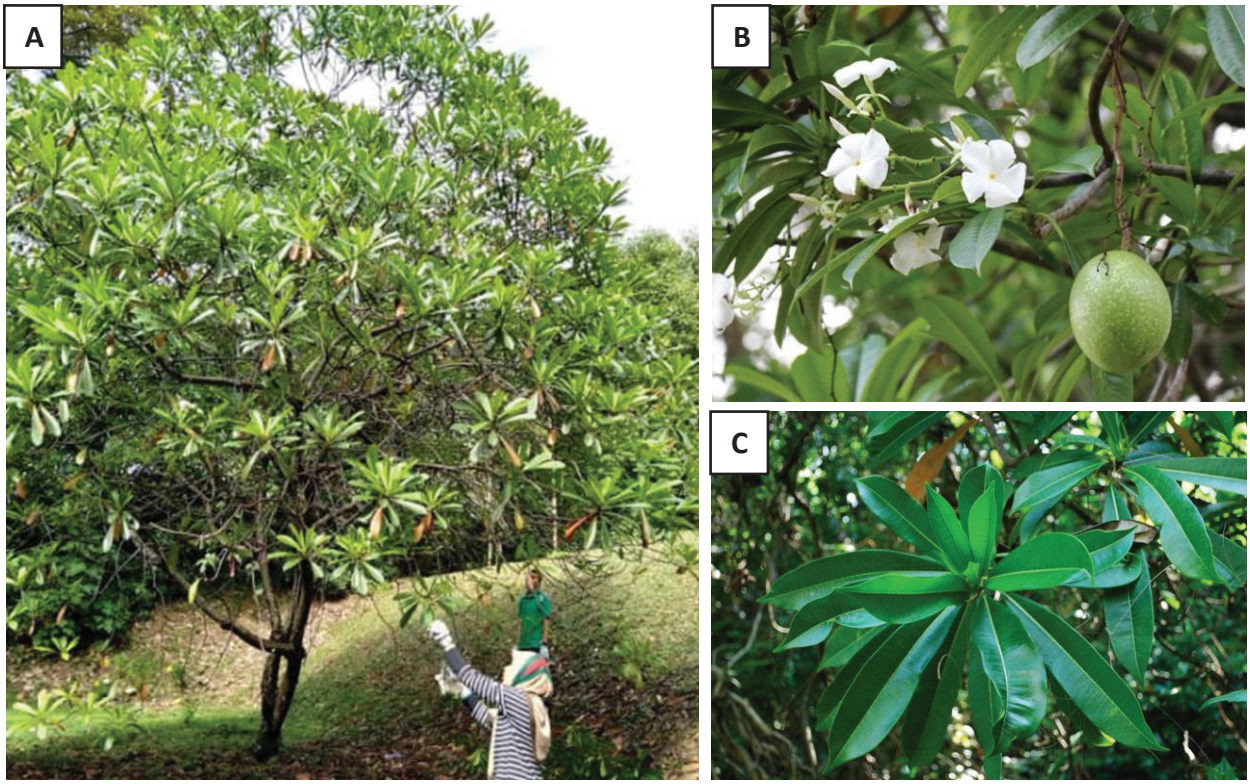


**Figure 1** *Cerbera odollam* tree (A), its flowers and fruit (B) and leaves (C)

## Cells viability assay

Cells were seeded at approximately 6000 cells per well of 96-well plates and allowed to grow for 24 hours. Each cell line was then treated with 17βH-neriifolin, digitoxin, digoxin, ouabain and paclitaxel at five different concentrations (0.4, 2, 10, 50 and 100 nM), with n ≤ 9. Additionally, normal liver (WRL-68) cells were treated with paclitaxel at concentrations of 32, 160, 800, 4000 and 20000 nM. Paclitaxel was used as the positive control drug. After 72 hours, treated cells were subjected for Sulforhodamine B (SRB) assay (Skehan et al. 1990). Briefly, treated cells were fixed with 50 µL of 50% (w/v) cold trichloroacetic acid (TCA) and incubated for 30 minutes at room temperature. TCA-fixed cells were rinsed with tap water and air-dried. The cells were then stained with 100 µL of 0.4% (w/v) SRB solution for 30 minutes and then rinsed with 1% acetic acid and air-dried. Finally, SRB-stained cells were solubilised with 100 µL of 10 mM Tris base for 5 minutes on a microplate shaker, and the optical density (OD) of treated and non-treated cells were read at 492 nm with a Magellan V 7.5 microplate reader (Tecan, Austria). The percentage of cell viability was calculated based on $OD_{492nm}$ of the treated cells/$OD_{492nm}$ of non-treated cells x 100. Half maximal inhibitory concentration ($IC_{50}$) was determined from the concentration-response curve of percentage of cells viability versus concentration (nM), based on the data obtained from three independent replicates. Compounds with $IC_{50}$ less than and/or equivalent to 50 µM is considered active (Boik 2001). All data were expressed as means ± standard error of the means (SEM).

## Selectivity index in relative to normal liver cell line

Selectivity index (SI) was determined by dividing the $IC_{50}$ value of a compound tested in normal cells by the $IC_{50}$ value in cancer cells. A higher SI value indicates greater specificity of the compound for cancer cells and reduced cytotoxicity toward normal cells. An SI value of ≥ 2 suggests therapeutic potential (Suffness & Pezzuto 1990).

## Statistical analysis

Statistical comparisons between treatment group and control group were performed using one-way ANOVA followed by Dunnett's test. This analysis was performed using GraphPad Prism software version 5.

## RESULTS

### *In vitro* cytotoxicity effects on cervical, lung and prostate cancers

The cytotoxic effects of 17βH-neriifolin and the cardiac glycosides digitoxin, digoxin and ouabain were tested against three cancer cell lines: HeLa, A549 and DU145. For comparative purposes, paclitaxel was also evaluated on its *in vitro* cytotoxicity as it is currently, one of the most utilised chemotherapeutic agents in the treatment of cervical, lung and prostate cancers (Gilligan and Kantoff, 2002, Della Corte et al. 2020). Clinically, paclitaxel was reported to have adverse effects (e.g. neurotoxicity, hypersensitivity reactions, haematological toxicity, gastrointestinal toxicity and cardiotoxicity) when given to cancer patients (Al-Mahayri et al. 2021). Here we report that 17βH-neriifolin was more cytotoxic in all cancer cells tested than ouabain, digitoxin, and digoxin with $IC_{50}$ values ranged from 8.36 ± 0.24 nM to 16.17 ± 1.33 nM, 10.15 ± 0.052 nM to 34.88 ± 2.11 nM, 21.61 ± 0.74 nM to 116.04 ± 8.68 nM, and 27.33 ± 0.23 nM to 604.65 ± 8.90 nM, respectively (Figure 2 and Table 1). Dunnett's test showing *p*-value less than 0.01 indicates that cardiac glycosides differed significantly from paclitaxel (Figure 3). All four cardiac glycosides showed less cytotoxic effect in normal liver (WRL-68) cell line. A normal liver cell line was incorporated in this study because liver acts as a first-line defense system against toxicity in the human body. The majority of drug withdrawals during drug development phase are due to the potential for severe liver injury (Regev 2014).

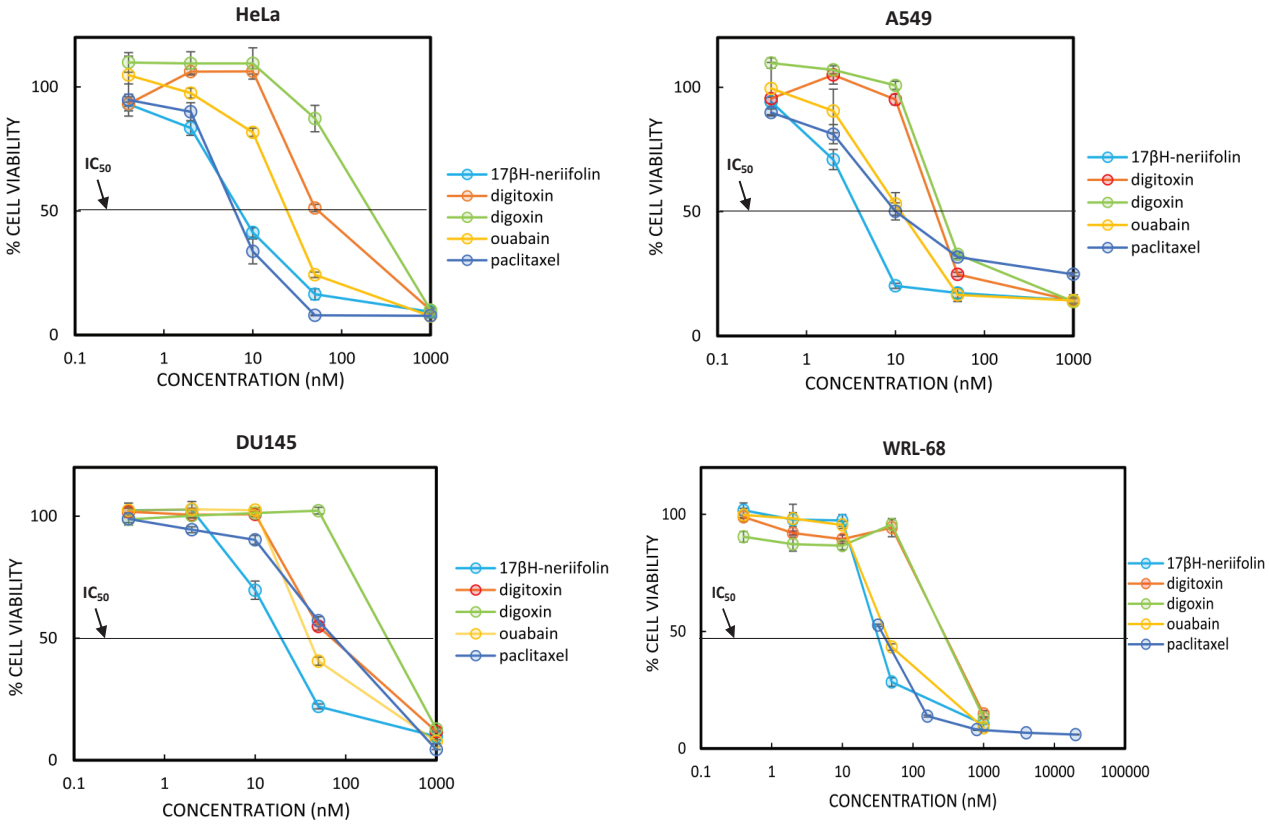


**Figure 2** Cell viability analysis in HeLa, A549, DU145 and WRL-68 cells. The cells were treated with various concentrations of compounds for 72 hours and cell viability was determined using the SRB assay

**Table 1** Cytotoxicity of 17βH-neriifolin, digitoxin, digoxin, ouabain and paclitaxel against human cancer cell lines and a normal cell line

| Cell line | $IC_{50}$ ± SEM in nM ($n \leq 9$) | | | | |
|---|---|---|---|---|---|
| | 17βH-neriifolin | Digitoxin | Digoxin | Ouabain | Paclitaxel |
| HeLa | 8.36 ± 0.24 | 58.82 ± 5.48 | 507.01 ± 38.26 | 19.16 ±0.55 | 7.71 ±0.56 |
| A549 | 5.28 ±0.43 | 21.61 ± 0.74 | 27.33 ± 0.23 | 10.15 ±0.052 | 9.53 ±0.41 |
| DU145 | 16.17 ±1.33 | 116.04 ± 8.68 | 604.65 ±8.90 | 34.88 ±2.11 | 192.66 ±8.57 |
| WRL-68 | 23.93 ± 1.42 | 579.00 ±22.43 | 575.67 ±10.27 | 37.48 ±2.01 | 40.77 ±1.50 |

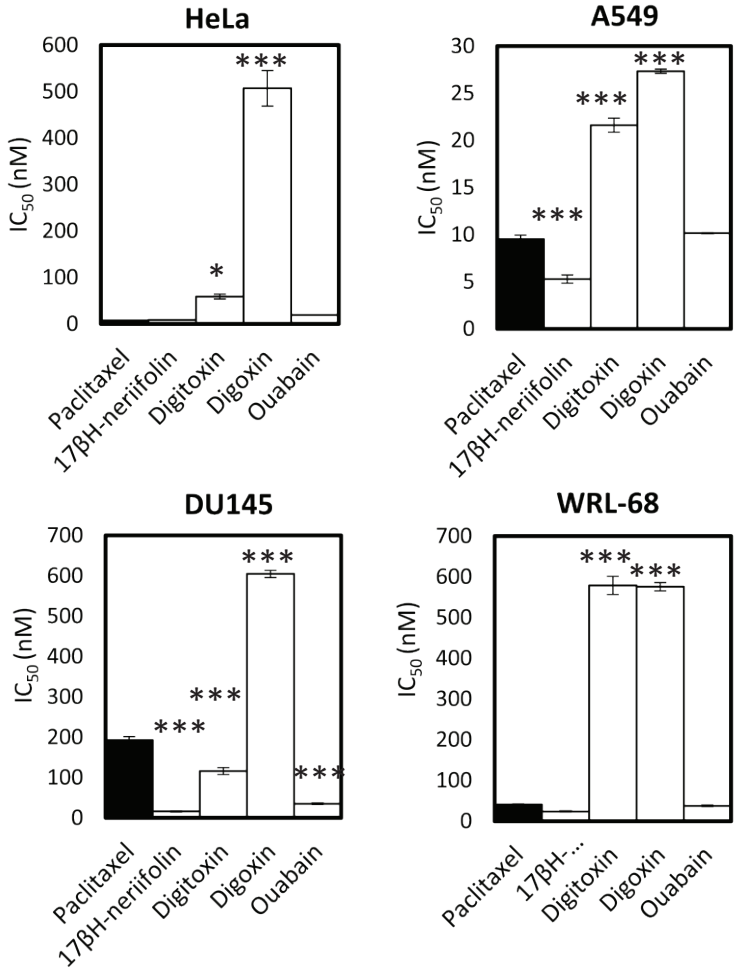


**Figure 3** Statistical analysis of 17βH-neriifolin and three cardiac glycosides in relative to the control group treated with paclitaxel. Significant differences of cardiac glycosides to paclitaxel are indicated by *$p < 0.01$, **$p < 0.001$ and ***$p < 0.0001$ (one-way ANOVA with Dunnett's test)

## Selectivity index

Selectivity index (SI) is a method for measuring potential anticancer compound with high selectivity which destroy cancerous cells without causing serious damage to normal cells. In this study, 17βH-neriifolin gave SI values ranging from 1.48 to 4.53, which were higher than the standard drug paclitaxel, with an SI range of 0.21 to 5.29 (Table 2). This data indicates that 17βH-neriifolin is more selective toward HeLa, A549 and DU145 cell lines than the WRL-68 cell line, and it demonstrates greater *in vitro* cytotoxic activity and selectivity for cancer cells compared to paclitaxel, highlighting its potential as a more effective therapeutic option over the clinically used paclitaxel.

## DISCUSSION

Previously, we reported that the cardiac glycosides 17βH-neriifolin and ouabain were cytotoxic to breast (MCF-7, T47D), colorectal (HT-29), ovarian (A2780, SKOV-3) and melanoma (A375) cancer cell lines, with $IC_{50}$ values (means ± SEM, nM) of 28 ± 1.0 and 53 ± 0.4, 26 ± 0.13 and 45 ± 1.2, 30 ± 1.8 and 62 ± 2.4, 22 ± 1.5 and 55 ± 0.26, 28 ± 1.2 and 56 ± 0.91 and 260 ± 16 and 42 ± 1.8, respectively (Siti Syarifah et al. 2014, Nurhanan et al. 2020). As a continuation of our study on these compounds, we present cytotoxicity data for 17βH-neriifolin, ouabain and two additional cardiac glycosides, i.e., digitoxin and digoxin on cervical (HeLa), lung (A549) and prostate (DU145) cancer cell lines. Our findings indicate that 17βH-neriifolin and ouabain are not selective in cytotoxicity among the nine cancer cell lines tested but were selectively cytotoxic against the normal liver cells (17βH-neriifolin and ouabain are more cytotoxic to cancer cells than the normal cells) compared to paclitaxel. Other CGs digitoxin and digoxin demonstrated cytotoxicity toward HeLa, A549 and DU145 cancer cells but their cytotoxic effects were not assessed in six other cancer cell lines (breast (MCF-7, T47D), colorectal (HT-29), ovarian (A2780, SKOV-3) and melanoma (A375)).

Several reports published over the last decade suggest that 17βH-neriifolin has significant *in vitro* anticancer potential. Isolated from the seeds of *Cerbera odollam*, 17βH-neriifolin, has been reported to exhibit cytotoxic effect against human small cell lung cancer (NCI-H187, $ED_{50}$: 0.076 µg/mL), oral human epidermoid carcinoma (KB, $ED_{50}$: 0.017 µg/mL) and human breast cancer cells (BC, $ED_{50}$: 0.048 µg/mL) (Laphookhieo et al. 2004). Besides *C. odollam*, 17βH-neriifolin has been isolated from the seeds, fruits and roots of *C. manghas*. Although no cytotoxicity data were reported for the seeds of *C. manghas* (Cheenpracha et al. 2004, Cao et al. 2013), the compound showed cytotoxic effects against a human acute myeloid leukemia (AML) cell line THP-1 with an $IC_{50}$ of 30 nM from the unripe fruits (Takase et al. 2022) and against a human colon cancer cell line (Col2) with an $IC_{50}$ of 0.02 µg/mL from the roots (Chang et al. 2000).

Stenkvist (2001) was the first to discover that cancer patients receiving cardiac glycosides medication for cardiac problems had a lower mortality rate compared to patients not receiving CGs treatment. Since this discovery, numerous independent studies have reported the *in vitro* anticancer effects of CGs on various cancer cell lines including cervical, lung and prostate cancer cells (Osman et al. 2017). Calderon-Montano et al. (2013) has shown that digitoxin, digoxin and ouabain induced *in vitro*

**Table 2** Selectivity index (SI) for 17βH-neriifolin, digitoxin, digoxin, ouabain and paclitaxel for cancer cell lines when compared to normal liver (WRL-68) cell line

| Cell line | 17βH-neriifolin | Digitoxin | Digoxin | Ouabain | Paclitaxel |
|---|---|---|---|---|---|
| HeLa | 2.86 | 9.84 | 1.14 | 1.96 | 5.29 |
| A549 | 4.53 | 26.79 | 21.06 | 3.69 | 4.28 |
| DU145 | 1.48 | 4.99 | 0.95 | 1.07 | 0.21 |

anticancer effects in human lung A549 cells after 48 hours of treatment, with $IC_{50}$ values of 7.39 ± 0.6 nM, 8.0 ± 1.3 nM and 5.3 ± 0.4 nM, respectively. Digitoxin, digoxin and ouabain were also shown to inhibit the proliferation of human prostate (LNCaP) cancer cells after 24 hours of treatment at $IC_{50}$ values of 1 to 10 µM, above 10 µM, and at 10 µM, respectively (Yeh et al. 2001). Other report suggests that digoxin induced cytotoxicity in human cervical (HeLa, $IC_{50}$: 151 nM) and lung (A549, $IC_{50}$: 31 nM) cancer cells (Pereira et al. 2019).

Cardiac glycosides work by inhibiting the $Na^+/K^+$- ATPase activity in both cardiac and cancer cells (Winnicka et al. 2006, Nurhanan et al. 2020, Salim et al. 2020). In prior work, 17βH-neriifolin was found to target $Na^+/K^+$- ATPase and inhibit the sodium-potassium pump through *in vitro* malachite green assay and *in silico* studies (Nurhanan et al. 2020). This inhibition increases intracellular sodium, followed by increased of intracellular calcium through the effect of $Na^+$, $Ca^{2+}$ exchanger. In cardiac, this mechanism can result in rapid heartbeats, while in cancer cells, it can trigger apoptosis. Although this mechanism is beneficial in treating certain heart conditions, its narrow therapeutic index—small margin between effectiveness and toxicity, can result in arrhythmia, which can be life-threatening if drug concentrations exceed the therapeutic range. Common symptoms of high doses include nausea, vomiting, gastrointestinal upset, and in rare cases, yellow bias in vision (xanthopsia) (Krantz 1958). Therefore, it is important to monitor plasma drug levels in treated cancer patients below those observed in the plasma of cardiac patients receiving the same medication.

Despite concerns about the narrow therapeutic index of cardiac glycosides, their potential in treating localised and advanced cancers cannot be overlooked, as several have been assessed in clinical trials. To date, 34 phase I and II clinical trials are currently being evaluated or have been completed for digoxin in treating cancer, both as a monotherapy or in combination with other drugs (Clinicaltrials.gov 2024). For instance, the phase II clinical trial for digoxin in the treatment of recurrent prostate cancer (ClinicalTrials.gov ID NCT01162135) has been completed, as well as phase I trial combining digoxin with vemurafenib for metastatic melanoma (ClinicalTrials.gov ID NCT01765569). Another phase II trial is recruiting volunteers to evaluate the combination of digoxin with metformin, simvastatin and gemcitabine to treat advanced pancreatic cancer (ClinicalTrials.gov ID NCT06030622).

It is hoped that 17βH-neriifolin, with its potent cytotoxicity and selectivity, can advance further in pharmacological and safety, potentially offering a new therapeutic approach for cancer treatment.

## CONCLUSION

Cardiac glycosides are promising anticancer drugs, with several undergoing or having completed phase I and II clinical trials for cancer treatment. We have recently shown that 17βH-neriifolin induces the killing of cervical, lung and prostate cancer cells at nanomolar concentrations, at concentrations lower than those required for digitoxin, digoxin and ouabain. Although we discuss the *in vitro* effects and their immediate relevance to therapeutic potential, *in vivo* data is essential for understanding both safety and efficacy in clinical applications. Given the established pharmacological and safety profiles of compounds like digitoxin and digoxin, it is vital to thoroughly investigate the pharmacological and safety profiles of 17βH-neriifolin through comprehensive preclinical and clinical studies.

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