POTENTIAL ANTICANCER COMPOUND FROM *CERBERA ODOLLAM*

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SITI SYARIFAH MM, NURHANAN MY, MUHD HAFFIZ J, MOHD ILHAM A, GETHA K, ASIAH O, NORHAYATI I, LILI SAHIRA H & ANEE SURYANI S. 2011. Potential anticancer compound from *Cerbera odollam*. The cytotoxicity of the leaf of *Cerbera odollam* was investigated against two breast cancer cell lines (T47D and MCF7), two ovarian cancer cell lines (SKOV3 and CaOV3) and a normal (Vero) cell line. The crude methanolic extract was subjected to liquid–liquid fractionation with ethyl acetate, butanol and water. Ethyl acetate fraction exhibited higher cytotoxic effect than methanol, butanol and aqueous fractions. Following bioassay guided isolation, 17βH-neriifolin was isolated as a potential anticancer agent from *C. odollam* leaf. It showed potent anticancer activity with IC_{50} values of 17, 21, 28, 32 and 24 nM against MCF7, T47D, SKOV3, CaOV3 and Vero cell lines respectively.

Keywords: Ovarian cancer, breast cancer, plant extracts, carcinoma cell lines, SRB assay

SITI SYARIFAH MM, NURHANAN MY, MUHD HAFFIZ J, MOHD ILHAM A, GETHA K, ASIAH O, NORHAYATI I, LILI SAHIRA H & ANEE SURYANI S. 2011. Sebatian antibarah yang berpotensi daripada *Cerbera odollam*. Sitotoksisiti daun *Cerbera odollam* dikaji terhadap dua titisan sel barah payudara (T47D dan MCF7), dua titisan sel barah ovari (SKOV3 and CaOV3) dan satu titisan sel normal (Vero). Ekstrak metanol *C. odollam* dipisahkan melalui pemeringkatan cecair–cecair dengan etil asetat, butanol dan air. Pecahan etil asetat menunjukkan kesan sitotoksisiti yang lebih kuat daripada pecahan metanol, butanol dan air. Melalui pemencilan berpandukan bioasai, 17βH-neriifolin yang berpotensi sebagai agen antibarah diasingkan daripada daun *C. odollam*. Ia menunjukkan aktiviti antibarah yang kuat dengan nilai IC_{50} 17 nM, 21 nM, 28 nM, 32 nM dan 24 nM masing-masing terhadap titisan-titisan sel MCF7, T47D, SKOV3, CaOV3 dan Vero.

INTRODUCTION

The potential of using natural products as anticancer agents was recognised in the 1950s by the US National Cancer Institute (NCI). Natural products have since made major contributions to the discovery of new anticancer agents (Cragg & Newman 2005). There is a long history of medicinal use of plants in South-East Asian countries, some of which have proven useful to humans as pharmaceuticals, which are relatively non-toxic, inexpensive and available in ingestive form (Gali-Muhtasib et al. 2006). In spite of the success of natural product for anticancer drug discovery, studies on plants for the treatment of cancer are rare in Malaysia. This makes Malaysia a promising site for discovery of novel biologically-active substances from its flora (Burkhill 1966, Perry & Metzger 1980, Murakami et al. 1999).

Cerbera odollam is a tree belonging to the family Apocynaceae. It is also known as pong-pong, buta-buta, nyan or yellow-eyed cerbera (Gaillard et al. 2004). It grows in coastal salt swamps and creeks in south India and along river banks in southern and central Vietnam, Cambodia, Sri Lanka, Myanmar and Malaysia (Chopra et al. 1958, Minh Hien et al. 1991). In South-East Asia, the oily seeds are mixed with other oils as an insect repellent or are burned for light. In some countries the leaves and barks are consumed for their cathartic properties (Gaillard et al. 2004). The latex is known in India for its emetic, purgative and

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irritant effects (Chopra et al. 1958). Nurhanan et al. (2008) reported the anti-proliferative effects of *C. odollam* methanol extract against human breast cancer, namely, MCF7 and T47D cell lines with IC_{50} values of 8.49 and 10.99 µg ml^{-1} respectively.

The aim of this study was to evaluate the cytotoxic effects of the extracts and bioactive compound isolated from the ethyl acetate (EtOAc) fraction of *C. odollam* leaves against human breast and ovarian cancer cell lines.

MATERIALS AND METHODS

Plant material

Leaf samples of *C. odollam* (3 kg) were collected from Rompin, Pahang, Malaysia. The plant sample was authenticated by a botanist and the voucher specimen deposited at the Forest Research Institute Malaysia.

General methods

Melting points were determined using an electrothermal melting point apparatus. The $[\alpha]_D$ values were determined with a digital polarimeter. Infra-red (IR) spectrum was recorded on a spectrophotometer. The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 400 and 100 MHz respectively in deuterated chloroform ($CDCl_3$) using tetra methyl silane (TMS) as an internal standard. Mass spectrometry value was obtained on liquid chromatography-mass spectrometry (LC-MS/MS) using electrospray ionisation (ESI) voltage of 4500 V. Vacuum liquid chromatography (VLC) and column chromatography (CC) were carried out using silica gel 230–400 mesh and 70–230 mesh respectively. The chemotherapy drug used was tamoxifen.

Extraction and isolation

Fresh leaves were cleaned and dried in an oven at 45 °C. The air-dried and ground leaves of *C. odollam* (450 g) were successively extracted in methanol (MeOH) (3.5 l) by Soxhlet extractor for 18 hours. The methanol extract was evaporated using rotary evaporator for five hours (yield 128.8 g, 28.62%) before it was partitioned successively with ethyl acetate (EtOAc) (500 ml), butanol (BuOH) (500 ml) and water (500 ml) in triplicates to yield EtOAc (7.4 g, 6.4%), BuOH (19.5 g, 16.8%), and H_2O (34.89 g, 30%) soluble fractions. The EtOAc and BuOH fractions were dried using rotary evaporator for one to three hours. The aqueous fraction was freeze dried for 72 hours before being subjected to cytotoxicity analysis. EtOAc-soluble fraction was fractionated by VLC on silica gel 60 (230–400 mesh) using *n*-hexane, *n*-hexane-EtOAc (9:1, 7:3, 4:6) and EtOAc-MeOH (8:2, 5:5, 1:9) as eluents to obtain 16 fractions (FRC 1–FRC 16). The combined active fractions, FRC 10 to FRC 12 (1.61 g) (Figure 1), were purified by CC on silica gel 70–230 mesh and eluted with n-hexane-EtOAc (9:1, 4:6, 1:9), EtOAc and EtOAc-MeOH (9:1, 7:3, 5:5) to obtain 17βH-neriifolin (0.0261 g, 0.006%).

Cytotoxicity assay

All extracts, fractions and isolated compound were assayed against five cell lines consisting of breast cancer (MCF7 and T47D), ovarian cancer (SKOV3 and CaOV3) and normal kidney (Vero) cells. These cell lines were purchased from the American Type Culture Collection (ATCC). The routine maintenance of the cell lines were performed according to the protocol from ATCC. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and antibiotics, 50 IU ml^{-1} penicillin and 50 µg ml^{-1} streptomycin.

For the cytotoxicity assay, 100 µl/well of the cell suspension were seeded in 96-well microtiter plates at a plating density of 1×10^5 cells ml^{-1} based on cell growth characteristics and incubated at 37 °C and 5% CO_2 to allow for cell attachment. After 24 hours, the cells were treated with the MeOH extract and the three fractions (EtOAC, BuOH and aqueous) (1% v/v) against four cancer cell lines and a normal cell line. From the results, the active fractions were those which gave IC_{50} values of less or equal to 10 µg ml^{-1} after 72 hours. The active fraction was then fractionated using VLC to obtain more fractions for compound identification.

Extract and fractions were dissolved in ethanol (1% v/v) and used for treating the cells. Cells without extract addition but treated with ethanol served as negative control and tamoxifen was used as positive control. Another set of control wells on the same plate contained cells and medium only. The samples were diluted in medium to produce

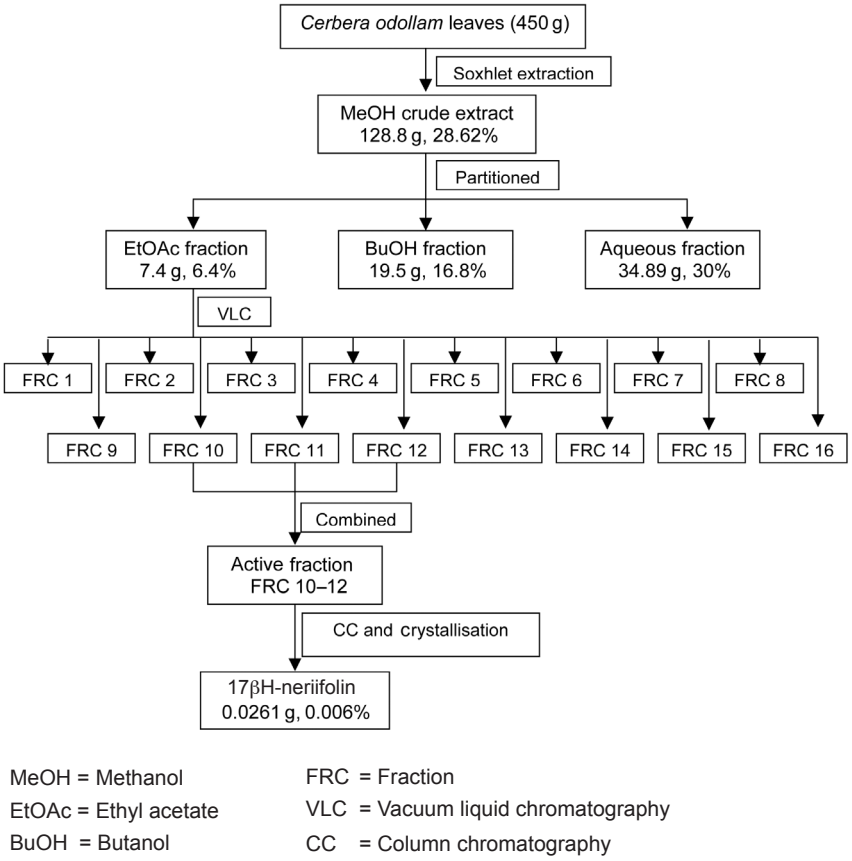

Figure 1 Extraction, fractionation, isolation and purification of 17βH-neriifolin from the *Cerbera odollam* methanol crude extract

five concentrations of 0.01, 0.1, 1.0, 10, 100 µg ml^{-1} and subjected to cytotoxicity assay to determine the IC_{50} values. An amount of 200 µl/well of each concentration was added to the plates in two replicates. The plates were incubated for 72 hours and assayed using the sulforhodamine (SRB) assay (Skehan et al. 1990, Itharat et al. 2004). The plates were then analysed using Elisa reader and software at 492 nm to determine the optical density (OD) which indicated the number of living cells. Three replicate plates were used to determine the cytotoxicity of each sample. Optical density values were used to calculate the percentage of living cells using the formula below.

$$\frac{\text{OD of ethanol-treated cells} - \text{OD of plant-treated cells}}{\text{OD of control cells} - \text{OD of ethanol-treated cells}} \times 100$$

According to the Food and Drug Administration, IC_{50} represents the concentration of the extract that is required for 50% inhibition of the cell population. In this study, the IC_{50} values of each extract against breast and ovarian cancer cell lines were calculated from the dose-response curve (percentage of living cells versus the extract concentration) (Nurhanan et al. 2008).

RESULTS AND DISCUSSION

Isolation and identification

The MeOH extract of the leaves of *C. odollam* exhibited cytotoxic effect against breast cancer (MCF7 and T47D) and ovarian cancer (SKOV3 and CaOV3) cell lines with IC_{50} values of ≤ 6.9 µg ml^{-1} (Table 1). The crude extract was successively partitioned using EtOAc, BuOH and water and the highest cytotoxic effect was found particularly in the EtOAc extract (IC_{50} ≤ 2.3 µg ml^{-1}). Thus, the EtOAc extract was subjected to several chromatographic techniques such as VLC and CC to yield 17βH-neriifolin.

By correlating with melting point, $[\alpha]_D$ value and spectral data (IR, ^{1}H, ^{13}C NMR and MS) of literature values (Abe & Yamauchi 1977, Yamauchi et al. 1987) the compound was identified as 17βH-neriifolin (Figure 2). 17βH-neriifolin was obtained as white solid with a molecular formula of $C_{30}H_{46}O_8$ on the basis of ESI-MS data ($[M+H]^+$ *m/z* 535.3 calcd 534.3193). The IR spectrum showed the presence of hydroxyl (3410 cm^{-1}) and carbonyl (1741 and 1715 cm^{-1}) groups. ^{1}H-NMR

Table 1 The cytotoxicity of *Cerbera odollam* methanol crude extract and the fractions against different cell lines

Cerbera odollam	Anti-proliferative activity IC_{50} ± SEM (µg ml^{-1})				
	MCF7	T47D	SKOV3	CAOV3	Vero
Methanol extract	6.4 ± 0.02	6.9 ± 0.05	3.3 ± 0.2	1.4 ± 0.01	1.4 ± 0.1
Ethyl acetate fraction	0.7 ± 0.08	2.3 ± 0.02	1.8 ± 0.06	0.9 ± 0.02	0.8 ± 0.2
Butanol fraction	≥ 50	≥ 50	≥ 50	≥ 50	≥ 50
Aqueous fraction	≥ 50	≥ 50	≥ 50	≥ 50	≥ 50

MCF7 and T47D = human breast adenocarcinoma cells; SKOV3 and CaOV3 = human ovarian cancer cells; Vero = normal kidney cell from African green monkey; n = 3

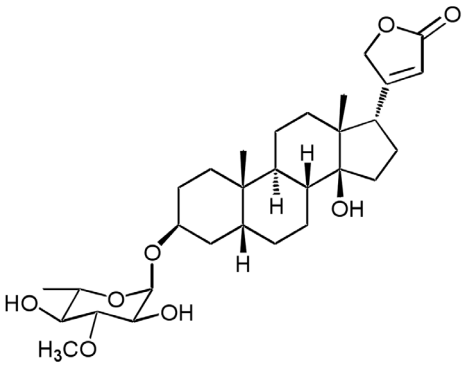

Figure 2 Isolated cardiac glycosides, 17βH-neriifolin from *Cerbera odollam* leaf

spectral data showed signals of cardenolide skeleton such as methylene protons C-21 (δ 4.96, 5.06, each, dd, *J*= 18.0, 1.2 Hz), an olefinic proton at C-22 (δ 5.92, s) and a methane proton at C-17 (δ 2.85, m). The ^{1}H- and ^{13}C-NMR spectral data showed one sugar molecule according to an anomeric proton at δ 4.79 (d, *J*= 4.0 Hz) for H-1' connected to an anomeric carbon at δ 98.1 from the heteronuclear multiple quantum coherence experiment. In the correlation spectroscopy experiment, the connectivity of all protons in sugar moiety was compared with 2'-*O*-acetyl-L-thevetose, the sugar moiety of cerberin (Abe & Yamauchi 1977). The proton signals of sugar moiety were shown at δ 3.44 (1H, dd, *J*= 9, 4 Hz, H-2'), δ 3.33 (1H, t, *J*= 9 Hz, H-3'), 3.03 (1H, t, *J*= 9 Hz, H-4'), 3.76 (1H, dq, *J*=9, 6 Hz, H-5'), 1.21 (3H, d, *J*=6.4 Hz, H-6') and 3.65 (3H, s, 3'-OMe). Two methyl singlets at δ 0.90 and 1.00 indicated 3H-18 and 3H-19 respectively. From the heteronuclear multiple bond correlation experiment, correlations of oxymethine proton, H-3 (δ 3.93) were observed with C-1 (30.7), C-1' (98.1) and C-5 (35.6); of H-17 (δ 2.85) with C-12 (40.2), C-15 (36.8) and C-21 (74.6), indicating that the α, β-unsaturated γ-lactone was located at C-17 (50.5) and the sugar moiety, at C-3 (75.0).

17βH-neriifolin was discovered in the leaves of *C. odollam* by Yamauchi et al. (1987) and in the leaves of *Cerbera manghas* by Abe and Yamauchi (1977). This compound has also been reported to be present in the seeds of *C. odollam* (Laphookhieo et al. 2004)

Even though 17βH-neriifolin is a known compound isolated from *C. odollam* leaves, there is no report on the cytotoxic effects of 17βH-neriifolin against ovarian cancer cell lines. From the report by Yamauchi et al. (1987), 17βH-neriifolin was not the only compound isolated from the leaves of *C. odollam*. Six minor and four major monosides were isolated from *C. odollam* and *C. manghas*. This paper discusses on the cytotoxic effect of 17βH-neriifolin against breast and ovarian cancer cell lines since only this compound was successfully isolated using the bioassay guided isolation technique.

Cytotoxicity

The assay results for the extracts summarised in Table 1 were separated into three categories: score 1—weak ($IC_{50} \geq 50$ µg ml^{-1}), score 2—moderately active (10 µg ml^{-1} < IC_{50} < 50 µg ml^{-1}) and score 3—active ($IC_{50} \leq 10$ µg ml^{-1}). An IC_{50} value of less or equal to 10 µg ml^{-1} was used as the cut-off point to select the potential fractions for further bioassay guided isolation.

Table 1 shows that the BuOH and aqueous fractions demonstrated weak toxicity against all cell lines, with IC_{50} values of ≥ 50 µg ml^{-1}. These fractions were eliminated based on the cut-off point whereby only IC_{50} value of less or equal to 10 µg ml^{-1} would be considered for further studies. The EtOAc fraction exhibited the highest cytotoxicity against MCF7 (IC_{50} 0.7 µg ml^{-1}), T47D (IC_{50} 2.3 µg ml^{-1}), SKOV3 (IC_{50} 1.8 µg ml^{-1}), CaOV3 (IC_{50} 0.9 µg ml^{-1}) and Vero (IC_{50} 0.8 µg ml^{-1}). This significant difference in IC_{50} values of EtOAc fraction has prompted us to carry out on-going work to determine the active ingredients that may have potential to be developed as valuable lead compound(s). This speculation, however, needs further studies for confirmation and therefore, EtOAc fraction was subjected to bioassay guided isolation.

Table 2 shows the cytotoxicity of the 16 fractions obtained from VLC fractionation of EtOAc fraction. To speed up the selection of active fraction, the 16 fractions were tested only against MCF7 and Vero cell lines. The MCF7 cell line was selected because it is the most widely used cell line in breast cancer research. According to Zhu et al. (2006), this cell line is a good model for identifying molecular event in some human cancers. Fractions 8, 10, 11, 12 and 13 demonstrated very active toxicity profiles against MCF7 cell line with IC_{50} values of 8.2, 0.071, 0.054, 0.07 and 7.64 µg ml^{-1} (Score 3) respectively (Table 2). Fraction 16 exhibited moderate activity with IC_{50} value of 21.42 µg ml^{-1} (Score 2). Other fractions showed weak anti-proliferative activities against MCF7 cell line (Score 1). The criterion of cytotoxicity for crude extracts as established by NCI is IC_{50} < 30 µg ml^{-1} (Suffness & Pezzuto 1990), while it is less than 4 µg ml^{-1} for pure compounds (Boik 2001). However, according to Boyd and Paull (1995), it depends on the objectives of the research when selecting the cut-off point in screening activity. Nurhanan (2008), who conducted a screening programme on 32 plant extracts, considered five plant extracts having IC_{50} < 30 µg ml^{-1} as active. However, to narrow down the selection of active extracts that have ultimate potential for biopharmaceutical use, the cut-off point used in this study was $IC_{50} \leq 10$ µg ml^{-1}. Factions 10, 11 and 12 showed IC_{50} values of less than 1 µg ml^{-1}. Based on the criterion set by Boik (2001) whereby IC_{50} values for pure compounds should be less than 4 µg ml^{-1}, these fractions were selected for further analysis.

Although it is recommended that the same non-tumour cell line be used when determining the selectivity index (SI) of the extracts, Vero cell line was used here to determine the *in vitro* cytotoxic effect as well as to establish the selectivity index of the extracts as an estimation of their therapeutic window instead of using normal breast and ovarian cell lines. Prayong et al. (2008) calculated the SI value of 14 plant species from the IC_{50} ratio in Vero cell versus malignant human hepatoma (HepG2). In our study, the SI value was calculated from the IC_{50} ratio of Vero cells versus MCF7 cells. The SI value indicates the selectivity of the samples towards tested cell lines.

In our anticancer screening activity, activity scores of the selectivity index value were separated into three categories: score 1—not selective ($SI \leq 1$), score 2—moderately selective ($1 < SI < 5$) and score 3—selective ($SI \geq 5$). None of the fractions showed SI score 3. Active fractions 10, 11 and 12 exhibited SI value of score 2. They were moderately selective against MCF7 cell line. The results were compared with the chemotherapy drug currently used for breast cancer patients, i.e. tamoxifen. The use of chemotherapy drugs is based on their selective toxicity towards malignant cells. Table 2 shows the IC_{50} value of score 3 for tamoxifen and the selectivity index value was grouped under the moderately selective group (score 2). Its bioactivity pattern showed similarity with fractions 10, 11 and 12.

Active fractions 10, 11 and 12 when combined and subjected to CC gave 17βH-neriifolin. Both compounds, tamoxifen and 17βH-neriifolin, were then subjected to cytotoxicity analysis. 17βH-neriifolin demonstrated higher cytotoxicity than tamoxifen with IC_{50} values < 32 nM against all the cell lines (Table 3). The differential toxicity exhibited by 17βH-neriifolin is highly significant and may warrant further active investigation.

Table 2 *In vitro* cytotoxic activity and the selectivity index values of the fractions against human breast cancer and normal cell lines

Ethyl acetate fraction	IC_{50} ± SEM (μg ml^{-1})		Activity score	SI value	SI score
	Cytotoxicity MCF7	Cytotoxicity VERO			
FRC 1	NT	NT	NT	NT	NT
FRC 2	NT	NT	NT	NT	NT
FRC 3	66.37 ± 0.17	80.23 ± 0.12	1	1.21	2
FRC 4	70.11 ± 0.12	72.93 ± 0.14	1	1.04	2
FRC 5	74.79 ± 0.05	>100	1	NV	NV
FRC 6	76.10 ± 0.08	82.6 ± 0.12	1	1.09	2
FRC 7	66.89 ± 0.07	71.46 ± 0.05	1	1.07	2
FRC 8	8.20 ± 0.13	7.95 ± 0.22	3	0.97	1
FRC 9	62.31 ± 0.2	62.17 ± 0.18	1	0.99	1
FRC 10	0.071 ± 0.1	0.075 ± 0.07	3	1.06	2
FRC 11	0.054 ± 0.04	0.063 ± 0.05	3	1.17	2
FRC 12	0.07 ± 0.02	0.073 ± 0.06	3	1.04	2
FRC 13	7.64 ± 0.02	7.52 ± 0.04	3	0.98	1
FRC 14	54.02 ± 0.1	71.79 ± 0.12	1	1.33	2
FRC 15	67.47 ± 0.14	76.9 ± 0.23	1	1.14	2
FRC 16	21.42 ± 0.05	44.73 ± 0.16	2	2.09	2
TMX	5.34 ± 0.07	5.8 ± 0.05	3	1.09	2

NT= not tested; NV= no value since highest concentration 100 μg ml^{-1} showed < 50% inhibition of growth; TMX = tamoxifen; n = 3

Activity score: 1 (weak) = $IC_{50} \geq 50$ μg ml^{-1}, score 2 (moderately active) = 10 μg $ml^{-1} < IC_{50} < 50$ μg ml^{-1}, score 3 (active) = $IC_{50} \leq 10$ μg ml^{-1}

SI value = $\frac{IC_{50} \text{ value of Vero cell}}{IC_{50} \text{ value of MCF7 cell}}$

SI score: score 1 (unselective) = SI ≤ 1, score 2 (moderately selective) = 1 < SI < 5, score 3 (selective) = SI ≥ 5

As the SI value exhibits differential activity of a pure compound, the greater the SI value the more selective it is (Koch et al. 2005). SI value that is less than 2 indicates general toxicity of the pure compound. The SI value for 17βH-neriifolin was found to range from 0.75 to 1.4 (Table 3). It is less than 2 and falls in between score 1 and 2. Likewise, the SI value for tamoxifen was also less than 2, ranging from 1.0 to 1.1. Badisa et al. (2009) reported that the SI value for 4-hydroxy tamoxifen, a known breast cancer chemodrug, was also less than 2. This suggests its general toxicity to the cells. The similarity of the SI value of 17βH-neriifolin and tamoxifen suggests that they may have the same target sites in the cells.

Cancer is an important cause of death in the world. Available treatments are costly and can cause other health problems. They vary in their effectiveness. For such reasons, the search for natural products having anticancer properties has been increasing in recent years (Galati & O'Brien 2004). New findings in the past five years have revealed the class of steroid-like compounds designated as cardiac glycosides which include well-known drugs such as digoxin, digitoxin and ouabain. These drugs are known to be involved in complex cell-signal transduction mechanisms, resulting in selective control of human tumour but not normal cellular proliferation (Newman et al. 2008).

In 2000, McConkey found that the cardiac glycosides induce apoptosis in androgen independent human prostate cancer cell lines *in vitro*. As such, they represent a promising form of targeted cancer chemotherapy. Since neriifolin is structurally related to the digitalis class of cardiac

Table 3 The cytotoxic activity of 17βH-neriifolin against panels of cell lines

Compound	Anti-proliferative activity IC_{50} ± SEM (nM)				
	MCF7	T47D	SKOV3	CaOV3	Vero
17βH-neriifolin	17 ± 0.02 (1.4)	21 ± 0.07 (1.1)	28 ± 0.22 (0.85)	32 ± 0.001 (0.75)	24 ± 0.21
Tamoxifen	13 700 ± 0.14 (1.05)	14 300 ± 0.02 (1.01)	13 200 ± 0.06 (1.1)	13 500 ± 0.03 (1.07)	14 500 ± 0.05

n = 3

Values in parentheses are SI (selective index) values.

$$\text{SI value} = \frac{IC_{50} \text{ value of Vero cell}}{IC_{50} \text{ value of MCF7/T47D/SKOV3/CaOV3 cell}}$$

glycosides, 17βH-neriifolin holds great potential as a valuable therapeutic agent against breast and ovarian cancers as single or adjuvant treatment. Reports claimed that cardenolides from *C. odollam* are widely used in the treatment of congestive heart failure and as antiarrhytmic agent (Newman et al. 2008). This group of compound may also serve as an effective agent for skin cancer treatment (Afaq et al. 2004). 17βH-neriifolin was also reported to show cytotoxic activities against human oral epidermoid carcinoma, human breast cancer and human small cell lung cancer cell lines (Cheenpracha et al. 2004, Laphookhieo et al. 2004).

One of the criteria in developing therapeutic drug for cancer is to minimise or have no side effects on the normal cells of cancer patients. Further studies are required for the development of synthetic or semi-synthetic 17βH-neriifolin as a novel therapeutic option for cancer.

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