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Selective cytotoxic activity of isolated compounds from *Globimetula dinklagei* and *Phragmanthera capitata* (Loranthaceae)

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Abstract: This study aimed to evaluate the selective cytotoxicity of six natural compounds on four cancerous cells (MCF-7, HeLa, Caco-2 and A549) and two normal intestinal and lung cells (Hs1.Int and Wl-38) cells. We also attempted to analyze basically the structure–activity relationships and to understand the mechanism of action of active compounds using the Caspase-Glo® 3/7 kit. Globimetulin B (**2**) isolated from *Globimetula dinklagei* was significantly cytotoxic on cancerous cells with 50% inhibitory concentrations (IC_{50}) ranging from 12.75 to 37.65 μM and the selectivity index (SI) values varying between 1.13 and 3.48 against both normal cells. The compound 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**5**) isolated from *Phragmanthera capitata* exhibited the highest cytotoxic activity on HeLa cells with the IC_{50} of 6.88 μM and the SI of 5.20 and 8.71 against Hs1.Int and Wl-38 cells, respectively. A hydroxyl group at C-3 of compounds was suggested as playing an important role in the cytotoxic activity. The induction of caspase-3 and -7 activity represents some proof that apoptosis has occurred in treated cells. Globimetulin B (**2**) selectively killed cancer cells with less toxicity to non-cancerous cells as compared to conventional doxorubicin therapy.

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Keywords: cancer; caspases; cytotoxicity; selectivity index; structure–activity relationship.

1 Background

Cancer is a leading cause of death in developing as well as developed countries. It is defined as a chronic disease characterized by abnormal and uncontrolled cell division. It represents a major public health problem around the world with 14.1 million new cancer cases diagnosed in 2012 and approximately 8.2 million deaths [1]. The five most frequent cancers identified are those of the lung, breast, colorectum, prostate and stomach [2]. Therapeutic approaches for cancer include surgery, radiotherapy and chemotherapy used alone or in combination. Chemotherapy is usually limited by various factors such as low response rates, drug resistance, and adverse effects that severely affect the quality of life of patients [3]. The new area of focus in the treatment of cancer is by administering drugs with selective toxicity. This approach aims to kill the growing cancer cells without affecting the normal human cells. Many side effects in chemotherapy are caused by this lack of selectivity of drugs, which target both normal and cancerous cells. In this sense, the search for new selective and effective therapies has recently gained momentum. Medicinal plant-based drug discovery remains an important area, where a systematic search may provide important leads against various pharmacological targets [4]. Medicinal plants are important for pharmacological research and the manufacture of drugs because their constituents can be used directly as therapeutic agents or indirectly as models in the synthesis of more pharmacologically active drugs.

The Loranthaceae, with about 77 genera and 950 species, is the largest family of the Santalales order, commonly named mistletoe [5]. All mistletoes are hemiparasitic plants depending on their host for water and minerals [6]. Plants from the Loranthaceae family are widely distributed through Africa, Asia, America and Europe. In Africa, this family is represented by 22 genera and about 250 species [7]. Loranthaceae plants are known

around the African continent as "*cure all*" and have been found beneficial as a remedy for many health problems. Leaves of these plants are used as a purgative and also for the treatment of mental conditions. Other parts of the plants are used as a blood purifier and for the treatment of cancer and gastrointestinal diseases [8, 9]. In Cameroon, the Loranthaceae are represented by 26 species belonging to 7 genera (*Agelanthus*, *Englerina*, *Helixanthera*, *Tapinanthus*, *Phragmanthera*, *Globimetula*, *Viscum*) [10, 11]. The genera *Phragmanthera* and *Globimetula* consist of eight and five species respectively and are widespread through the country. Previous phytochemical analysis revealed that triterpenoids, flavonoids, phenolics and glycosides are the main secondary metabolites present in *Globimetula dinklagei* and *Phragmanthera capitata* [12, 13]. One natural lactone isolated from leaves of *P. capitata* exhibited good antiplasmodial activity against the *Plasmodium falciparum* chloroquine-sensitive strains NF54 and 3D7 [13]. From the CH_2Cl_2/MeOH extract of the entire plant of *G. dinklagei*, four compounds were isolated: globimetulin A (**1**), globimetulin B (**2**), globimetulin C (**3**) [14] and 3-O-β-D-glucopyranosyl-α-amyrin (**4**) [15]. From the methanolic extract of the leaves of *P. capitata*, we isolated 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**5**) [16] and apigenin-8-C-*β*-D-glucopyranoside (**6**) [17]. The present study aims to evaluate the selective cytotoxicity of these isolated compounds on four carcinoma cell lines compared with two normal cell lines. A basic analysis of the structure–activity relationship (SAR) of the compounds is discussed in order to determine the chemical groups that may be responsible for the cytotoxicity activity. An attempt to understand the potential mechanism of action of the most active compounds was addressed by evaluating their effect on the induction of apoptosis.

2 Materials and methods

2.1 Plant material

The entire plant of *G. dinklagei* was harvested on *Manihot esculenta* (Euphorbiaceae) in October 2012 in Bandjoun (Cameroon). A voucher specimen was authenticated by Mr. Jiofack, plant taxonomist and deposited at the Cameroon National Herbarium under the number N°9960/SFR/CAM. The leaves of *P. capitata* were collected on *Pachyra insignis* (Bombacaceae) in April 2013 in Yaounde (Cameroon) and identified by comparison to the sample deposit under the number N°24673/SFR/CAM at the Cameroon National Herbarium.

2.2 Extraction procedure, isolation and characterization of compounds

Air-dried and ground shoot plants of *G. dinklagei* (2 kg) were extracted with a mixture of methylene chloride and methanol (CH_2Cl_2:MeOH, 1:1) at room temperature to yield 120 g of a greenish oily extract. This extract was then subjected to column chromatography (CC) eluting with gradient of *n*-hexane–EtOAc to give five fractions: F_1–F_5. Fraction F_3 (20 g of the most active fraction) was submitted to CC and eluted with gradient *n*-hexane–EtOAc to yield 45 sub-fractions of 300 mL each. Sub-fractions with similar thin layer chromatography (TLC) profiles were combined to give eight series (S_1–S_8). S_6 (6.3 g) was subjected to CC to give globimetulin A **1** (2 g) using 15% *n*-hexane–EtOAc, S_8 (2.1 g) was also submitted to CC to afford globimetulin C **3** (14 mg) using 20% *n*-hexane–EtOAc. Successive CC was applied to F_4 (22 g) to yield six subseries (E_1–E_6). E_4 (3 g) was subjected to successive preparative TLC to yield globimetulin B **2** (5 mg) using 30% *n*-hexane–EtOAc. Fraction F_5 (15 g) was submitted to successive CC and eluted with gradient of *n*-hexane–EtOAc to give 10 series S_9–S_{18}. S_{14} (1.2 g) was subjected to successive CC to afford the compound 3-O-β-D-glucopyranosyl-α-amyrin **4** (30 mg).

Air-dried and powdered leaves of *P. capitata* (2.3 kg) were macerated with hot methanol (MeOH), followed by filtration and evaporation of the solvent in vacuo to yield 210 g of a greenish extract. Two hundred grams of this extract was then dissolved in ethyl acetate (EtOAc), and after filtration and evaporation, we obtained 23 g of ethyl acetate extract. This extract was then subjected to silica gel open column chromatography eluted with a gradient of *n*-hexane–EtOAc. We collected 200 fractions of 150 mL each. After monitoring with thin layer chromatography, these fractions were grouped as F_1–F_{10}. Fraction F_6 (0.8 g) was submitted to CC eluted with 20% *n*-hexane–EtOAc to obtain 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin **5** (18 mg). Fraction F_{10} was further purified by CC eluted with EtOAc to yield apigenin-8-C-*β*-D-glucopyranoside **6** (35 mg). The structure of the isolated compounds was determined using spectroscopic techniques such as mass spectrometry (MS) and one- and two-dimensional nuclear magnetic resonance (1D and 2D NMR), and by comparison with data from literature.

2.3 Cell culture

The four cancer cell lines (MCF-7: human breast adenocarcinoma cells; HeLa: human cervix adenocarcinoma cells; Caco-2: human epithelial colorectal

adenocarcinoma cells; A549: human epithelial lung adenocarcinoma cells) and two normal human cell lines (Hs1. Int: human intestinal fibroblast, Wl-38: human lung fibroblast) were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were grown at 37 °C with 5% CO_2 in a humidified environment in Dulbecco's modified Eagle's medium high glucose (4.5 g/L) containing L-glutamine (4 mM) and sodium-pyruvate (Hyclone™, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (Capricorn Scientific Gmbh, Ebsdorfergrund, Germany).

2.4 Cell treatment and cytotoxicity assay

The cells were seeded at a density of 10^4 cells per well on 96-well microtiter plates, and they were incubated overnight at 37 °C with 5% CO_2 to allow the attachment of cells. After this, the cells were treated with different concentrations (100, 75, 50, 25, 10 and 5 µg/mL) of the compounds dissolved in dimethylsulfoxide (DMSO) and further diluted in fresh culture medium. In each experiment, the maximal concentration of DMSO (negative control) in the medium was 0.5%. After incubation for 48 h at 37 °C with 5% CO_2, the culture medium was discarded and replaced by fresh medium (200 µL) with 30 µL of thiazolyl blue tetrazolium bromide (5 mg/mL) dissolved in phosphate buffered saline. After incubation for 4 h, the medium was gently aspirated and the formazan crystals were dissolved in 50 µL of DMSO and kept in the dark for 15 min at room temperature. The absorbance was measured spectrophotometrically at 570 nm on a microplate reader (Synergy Multi-Mode Reader, BioTek, Winooski, VT, USA).

The viability rate of treated cells with the compounds was calculated for each concentration compared with the negative control. The 50% inhibitory concentrations (IC_{50}) for cancerous cells and the 50% lethal concentrations (LC_{50}) for non-cancerous cells were determined by plotting the linear regression curve of percentage of cell survival versus the logarithm of concentrations. The selectivity index (SI) values were calculated for each compound by dividing the LC_{50} of each normal cell by the IC_{50} of cancer cells in the same units.

2.5 Evaluation of the induction of apoptosis on treated cells

The induction of apoptosis by the most active compounds, globimetulin B (**2**) and 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**5**), was analyzed by measuring the caspase 3/7 activity on different cancer cell lines with the Caspase-Glo® 3/7 kit (Promega, Madison, WI, USA). All the four cancer cell lines were seeded at a density of 10^4 cells per well on 96-well microtiter plates and were allowed to adhere overnight. These cells were treated with these compounds at different concentrations ($½ \times IC_{50}$, IC_{50} and $2 \times IC_{50}$) or DMSO (0.5%) as negative control and were incubated at 37 °C with 5% CO_2 for 24 h. After treatment, the Caspase-Glo® 3/7 kit was prepared according to the manufacturer's guidelines and 100 µL of the reagent was added per well and incubated for 1 h at room temperature in the dark. Following this incubation, the luminescence was measured on a microplate reader (Synergy Multi-Mode Reader, BioTek, Winooski, VT, USA). The data were analyzed and expressed as percentage of the untreated cells (control) and fold change.

2.6 Statistical analysis

All experiments were performed in triplicate, and the results are presented as mean ± standard deviation (SD) values. Statistical analysis was carried out with GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA). The Student–Newman–Keuls test was used to determine p-values for the differences observed between tested compounds at the same concentration, while Dunnett's test was used to compare the test compounds with the control. Results were considered significantly different when $p \leq 0.05$.

3 Results

3.1 Identification and characterization of isolated compounds

The chemical characterization of the six natural compounds isolated from *G. dinklagei* and *P. capitata* is presented below and their structures are shown in Figure 1.

Compound 1 [globimetulin A: $C_{52}H_{90}O_3$, fast atom bombardment (FAB)-MS mass to charge ratio (m/z) 763.6945] was isolated from *G. dinklagei* as a colorless powder. ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectra showed 42 carbon signals corresponding to 7 methyls (δ_C 8.3, 10.8, 15.6, 16.4, 18.9, 19.4, 27.9); 8 methines (δ_H 37.4, 47.6, 48.1, 50.2, 52.0, 67.9, 72.4, 80.1); 20 methylenes with a $(CH_2)_n$ between 20.1 and 29.7 belonging to a long aliphatic chain; one sp^2 methylene at δ_H 109.7; five sp^3 quaternary carbons

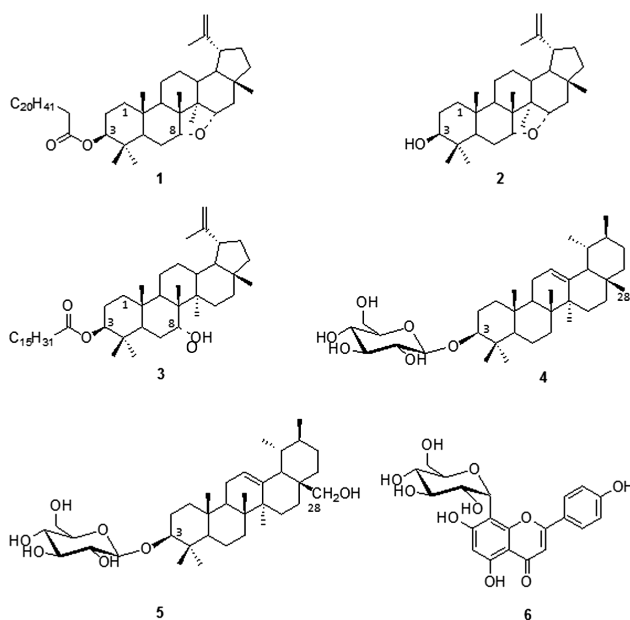

Figure 1: Chemical structures of globimetulin A **1**, globimetulin B **2**, globimetulin C **3**, 3-O-β-D-glucopyranosyl-α-amyrin **4** isolated from *Globimetula dinklagei*, 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin **5** and apigenin-8-C-β-D-glucopyranoside **6** isolated from *Phragmanthera capitata*.

(δ_H 37.2, 37.6, 42.5, 47.8, 48.9), an olefinic sp^2 carbon (δ_H 150.3) and a carbonyl of ester (δ_H 173.6). ^{1}H NMR and heteronuclear single quantum coherence data showed signals for an isopropenyl group at δ_H 1.69 (3H, s)/δ_C 19.4; δ_H 4.69 (1H, s)/δ_C 109.7; δ_H 4.60 (1H, s)/δ_C 109.7 and δ_C 173.6; 3 oxymethine protons at δ_H 4.47 (1H, dd)/δ_C 80.1, δ_H 3.80 (1H,dd)/δ_C 72.4, δ_H 4.15 (1H, dd)/δ_C 67.9; 6 signals of methyls at δ_H 0.86/δ_C 27.9, δ_H 0.85/δ_C 15.6, δ_H 0.84/δ_C 16.4, δ_H 1.08/δ_C 10.8, δ_H 0.99/δ_C 8.3, δ_H 0.83/δ_C 18.9. The triterpenoid skeleton of compound **1** was matching with 7β,15α-dihydroxy-lup-20(29)-en-3β-O-eicosanoate [15]. The high-resolution-FAB-MS spectrum of compound **1** showed the pseudomolecular ion peak at m/z 763.4266, containing eight degrees of unsaturation. The only difference with the two compounds is the presence in compound **1** of one supplementary unsaturation. To complete this remaining unsaturation, the two protons of oxymethines at δ_H 3.80 (1H, dd)/δ_C 72.4, δ_H 4.15 (1H, dd)/δ_C 67.9 are engaged in a tetrahydrofuran ring formed between the C-7 carbon and the oxygen atom, and between the C-15 carbon and the same oxygen atom. This was confirmed by the correlations observed on the heteronuclear multiple bond correlation spectrum between the proton at 3.80 with carbons at δ_C 52.0 (C-5), δ_C 48.9 (C-8), δ_C 28.1 (C-6), and the other proton at δ_H 4.15 with the carbon at δ_C 47.8 (C-14), δ_C 45.7 (C-16), δ_C 42.5 (C-27).

Compound 2 [globimetulin B: $C_{30}H_{48}O_2$, electrospray ionization (ESI)-MS m/z 440.3642] was isolated as an amorphous colorless powder. As compound **1**, compound **2** is a lupan-type triterpene with an additional tetrahydrofuran ring. The IR spectrum showed signals of a hydroxyl group at 3402 cm^{-1}. The ^{13}C NMR spectrum coupled with DEPT spectra showed signals of 7 methyls, 8 sp3 methylenes, 8 sp^3 methines, 2 sp^2 carbons and 5 sp^3 quaternary carbons. The only difference between the two compounds is the absence of the signals of the ester moiety replaced by the hydroxyl group. This is confirmed by the deshielding of the signal of H-3, which passed from δ_H 4.47 for compound **1** to δ_H 3.20 for compound **2**.

Compound 3 (globimetulin C: $C_{46}H_{80}O_3$, FAB-MS m/z 681.6165) was obtained from *G. dinklagei* as a colorless amorphous solid. A hydroxyl group and a carbonyl of ester appeared on the IR spectrum respectively at 3560.3 cm^{-1} and 1716.5 cm^{-1}. The ^{13}C NMR spectrum sorted by the DEPT spectrum showed signals of seven methyls, two olefinic carbons and two oxygenated methylenes including a $(CH_2)_n$ between δ_C 28.9 and δ_C 29.7. Like compounds **1** and **2**, compound **3** is a lupan triterpene with a palmitate moiety at C-3 [15]. The proton of the oxymethine appearing in the ^{1}H NMR spectrum at δ_H 3.83 is linked to the hydroxyl. This was confirmed by the cross-peak observed between this proton and carbons at δ_C 52.3 (-5), δ_C 46.7 (C-8), δ_C 10.1 (C-26), indicating its position at C-7.

Compound 4 (3-O-β-D-glucopyranosyl-α-amyrin: $C_{36}H_{60}O_6$, ESI-MS m/z 588) was isolated from *P. capitata* as white crystals. It gave a positive result to the Mölisch test, indicating the presence of a sugar moiety. The ^{13}C NMR spectra present signals of an urs-12-ene skeleton that is essentially two olefinic carbons and eight methyl groups. In addition, we observed signals of a sugar moiety at δ_H 4.47/δ_C 104.2, δ_H 4.24/δ_C 73.8, δ_H 3.14/δ_C 71.7, δ_H 3.40/δ_C 66.5, δ_H 4.33/δ_C 63.3, δ_H 3.27/δ_C 76.4; these signals are characteristic of a glucose. This substituent is at C-3 along with the proton of the oxymethine at δ_H 3.14 (1H, dd, 5; 5)/δ_C 72.6 [15].

Compound 5 (3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin: $C_{36}H_{60}O_7$, liquid chromatography (LC)-MS m/z 604) was isolated from *P. capitata* as white crystals. It gave a positive result to the Mölisch test, indicating the presence of a sugar moiety. The ^{1}H and ^{13}C NMR spectra present signals of an urs-12-ene skeleton and a glucose moiety like compound **4**; the main difference is the presence of a hydroxymethylene at 3.40 (2H, s)/63.6 in place of a methyl. The position of this hydroxyl group was determined by comparison with literature data to be in C-28 [18].

Compound 6 (8-C-β-D-glucopyranoside apigenin: $C_{21}H_{20}O_{10}$, ESI-MS m/z 433.47) was obtained from *P. capitata*

as a white powder. The positive result to the Shinoda and $FeCl_3$ tests indicates respectively the presence of the flavonoid and phenolic hydroxy group. Both ^{1}H and ^{13}C NMR spectra showed signals of apigenin and a glucose moiety [17]. The position of the sugar is at C-8, due to the correlation observed between the anomeric proton and carbon at 104.1 (C-8), 161.5 (C-7), 155.8 (C-9). $C_{21}H_{20}O_{10}$, ESI-MS m/z 433.47.

The chemical characterization of the six natural compounds isolated from *G. dinklagei* and *P. capitata* has been previously reported [14], and their structures are shown in Figure 1. Globimetulin A (**1**): colorless amorphous powder, $C_{52}H_{90}O_3$, FAB-MS m/z 763.6945; globimetulin B (**2**): colorless amorphous powder, $C_{30}H_{48}O_2$, ESI-MS m/z 440.3642; globimetulin C (**3**): colorless amorphous powder, $C_{46}H_{80}O_3$, FAB-MS m/z 681.6165 [14]; α-amyrin-3-O-β-D-glucopyranoside (**4**): colorless amorphous powder, $C_{36}H_{60}O_6$, ESI-MS m/z 588 [15]; 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**5**): colorless amorphous powder $C_{36}H_{60}O_7$, LC-MS m/z 604; apigenin-8-C-β-D-glucopyranoside (**6**): yellow powder, $C_{21}H_{20}O_{10}$, ESI-MS m/z 433.47 [17].

3.2 Cytotoxic effect of isolated compounds against different cell lines

The cell viability was determined using the methyl thiazolyl tetrazolium colorimetric assay and expressed as percentage of untreated cells (control). The compounds induced a concentration-dependent decrease in the percentage of cell survival (Figure 2). It is observed from this figure that compounds **2**, **3, 4** and **5** were significantly ($p < 0.05$) cytotoxic to all the cell lines. The most cytotoxic effect was exhibited by compound **2**, which caused almost 100% cell death of three cancer cells (MCF-7, HeLa and Caco-2) at concentrations of 100, 75 and 50 μg/mL. Based on the results shown in Figure 2, the IC_{50} were determined and are presented in Table 1. The results indicate that compound **2** was significantly ($p < 0.05$) cytotoxic on all the cancer cells with IC_{50} varying between 5.61 and 16.57 μg/mL (i.e. 12.75 and 37.65 μM, respectively). Compound **2** was moderately cytotoxic to both normal intestinal and lung cells (Hs1.Int and Wl-38) with the LC_{50} of 19.56 μg/mL (i.e. 44.45 μM) and 18.77 μg/mL (i.e. 42.65 μM), respectively. The determination of the SI showed the lowest toxic effect of compound **2** on normal cells compared to cancer cells. The SI values of compound **2** against both normal human cells varied between 1.13 and 3.48, which is above 1, and it therefore suggests that this compound can selectively kill cancer cells at the IC_{50} value without causing a harmful effect on normal human cells. In addition, when compared with the positive control (doxorubicin), compound **2** had the higher selectivity index against all cancer cell lines except A549 cells. On the other hand, compound **5** isolated from *P. capitata* was less cytotoxic to all cancer cell lines than compound **2** isolated from *G. dinklagei*, except against the human cervix cancer cells (HeLa) where compound **5** had an IC_{50} of 4.16 μg/mL (i.e. 6.88 μM). Compound **5** afforded the highest selectivity index in HeLa cells with the SI of 5.20 and 8.71 against normal intestinal and lung cells (Hs1.Int and Wl-38), respectively. As compounds **2** and **5** were found to exhibit good selective cytotoxicity against cancer cells, they were tested for 12, 24 and 48 h on different cancer cells at their respective IC_{50}, and these compounds induced a cytotoxic effect in a time-dependent manner (Figure 3). In addition, we found that these two compounds strongly inhibit the growth of HeLa cells with time. It therefore suggests that these compounds can be used as potential candidates for the development of drugs against cancer.

3.3 Structure–activity relationship of isolated compounds

In this study, the six compounds isolated from *G. dinklagei* and *P. capitata* were classified as triterpenoids (compounds **1**, **2** and **3**), saponins (compounds **4** and **5**) and flavonoid (compound **6**). It was found that compound **2** (globimetulin B) exhibited the highest activity, since the esterification of the hydroxyl group at C-3 has a mainly negative effect on cytotoxicity (Figure 1). It was also observed that the cytotoxic effect varied depending on the length of the carboxyl group attached to the hydroxyl group at position C-3. This is illustrated by the fact that compound **3** (globimetulin C), which has the shorter carboxyl group ($C_{15}H_{31}COO-$), was significantly ($p < 0.05$) more cytotoxic than compound **1** (globimetulin A), which has the longer carboxyl group ($C_{21}H_{43}COO-$). The cytotoxic effect of compound **3** may also be attributed to the presence of the free hydroxyl group at position C-8, which is present in compound **1** in an ether linkage. From these three triterpenoids, we observed that the cytotoxic effect of a compound increased with its polarity. Globimetulin B (**2**) is more polar than globimetulin C (**3**) and globimetulin A (**1**), which is the less polar compound. This was also seen with the two saponins (3-O-β-D-glucopyranosyl-α-amyrin **4** and 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin **5**), which are similar in their chemical structure except for the presence of the hydroxyl group at position C-28. The only flavonoid (apigenin-8-C-*β*-D-glucopyranoside **6**)

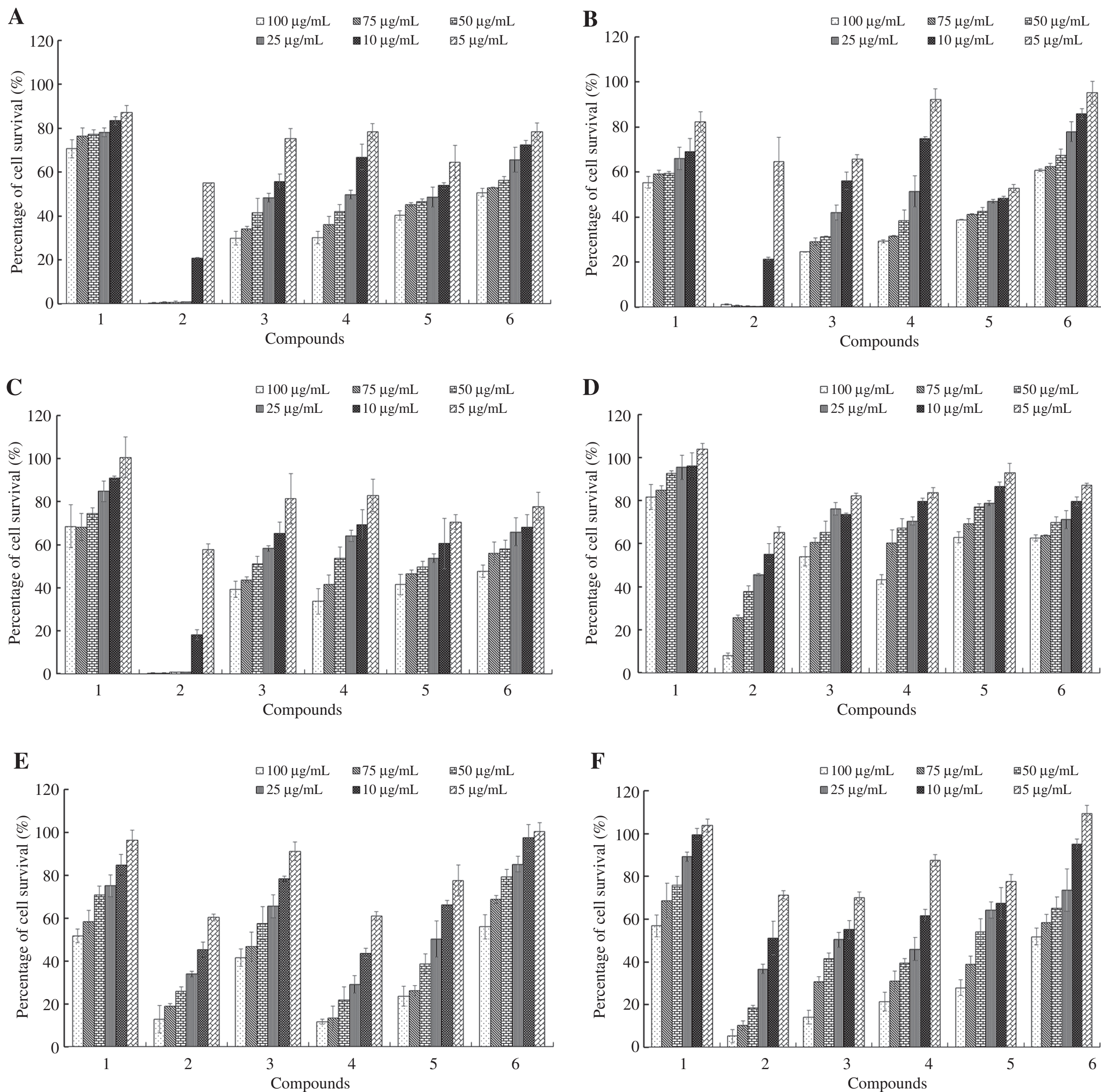

Figure 2: Growth inhibition of isolated compounds from *Globimetula dinklagei* and *Phragmanthera capitata* against different cell lines MCF-7 (A), HeLa (B), Caco-2 (C), A549 (D), Hs 1. Int (E) and Wl-38 (F) cell lines. Data are presented as mean ± SD of three independent experiments.

containing a sugar linkage had low cytotoxicity to all the cancer cells.

3.4 Compounds 2 and 5 induce caspase-dependent apoptosis in different cancerous cells

The analysis of the induction of caspsase-3 and -7 was carried out on the cancerous cells after 24 h of treatment with the most cytotoxic compounds (**2** and **5**) in order to understand the process of cell death caused by these compounds. The activation of caspase-3 and -7 enzymes was observed in all the four cancer cells treated with the compounds compared to the untreated controls (Figure 4). The data from this figure demonstrate that caspase-3 and -7 activities already increased significantly ($p < 0.05$ or $p < 0.01$) in MCF-7 and A549 cells at the concentration of $½ \times IC_{50}$ (1.3- to 1.5-fold change), and this activity was maintained at other concentrations (IC_{50} and $2 \times IC_{50}$).

Table 1: Cytotoxic activity (LC_{50} and IC_{50}) and the selectivity index (SI) of isolated compounds from *Globimetula dinklagei* and *Phragmanthera capitata* and reference drug (doxorubicin) against cancer cell lines.

Compounds	Cell lines	Hs 1. Int	Wl-38	MCF-7			HeLa			Caco-2			A549		
	Units	LC_{50}	LC_{50}	IC_{50}	$SI_{(1)}$	$SI_{(2)}$	IC_{50}	$SI_{(1)}$	$SI_{(2)}$	IC_{50}	$SI_{(1)}$	$SI_{(2)}$	IC_{50}	$SI_{(1)}$	$SI_{(2)}$
1	µg/mL	>100	>100	>100	nd	nd	>100	nd	nd	>100	nd	Nd	>100	nd	nd
	µM	>131.06	>131.06	>131.06			>131.06			>131.06			>131.06		
2	µg/mL	19.56±1.58[a]	18.77±1.42[a]	6.88±0.26[a]	2.84	2.73	5.61±2.48[a]	3.48	3.34	5.76±0.19[a]	3.39	3.25	16.57±1.61[a]	1.18	1.13
	µM	44.45±3.59	42.65±3.22	15.63±0.59[a]			12.75±5.63[a]			13.09±0.42[a]			37.65±3.66[a]		
3	µg/mL	21.81±1.98[a]	33.28±2.67	31.15±2.37	0.70	1.07	22.50±1.34	0.96	1.48	64.33±1.29	0.34	0.51	>100	<0.21	<0.33
	µM	32.02±2.90	48.86±3.92	45.80±3.48			33.08±1.97			94.61±1.89			>147.05		
4	µg/mL	15.99±0.86[a]	17.48±1.38[a]	45.18±0.94	0.35	0.38	49.02±0.86	0.32	0.35	61.13±5.46	0.26	0.28	88.34±0.28	0.18	0.19
	µM	27.19±1.46	29.72±2.34	76.83±1.59			83.36±1.46			103.97±9.28			150.23±0.47		
5	µg/mL	21.64±2.06[a]	36.23±1.74	42.54±9.89	0.51	0.85	4.16±1.46[a]	5.20	8.71	59.64±9.38	0.36	0.60	>100	<0.21	<0.36
	µM	38.54±1.59	59.98±2.88	70.43±16.37			6.88±2.41[a]			98.74±15.53			>165.56		
6	µg/mL	>100	>100	97.54±3.17	>1.02	>1.02	>100	nd	nd	96.07±3.67	>1.02	>1.02	>100	nd	nd
	µM	>230.94	>230.94	225.26±7.32			>230.94			221.87±8.47			>230.94		
Doxorubicin	µg/mL	1.14±0.25[b]	2.55±0.76[b]	1.03±0.33[b]	1.10	2.47	1.11±0.24[b]	1.03	2.29	1.74±0.19[b]	0.65	1.46	0.60±0.50[b]	1.66	4.25
	µM	2.09±0.45[b]	4.69±1.13[b]	1.78±0.58[b]			1.92±0.42[b]			3.00±0.34[b]			1.05±0.87[b]		

IC_{50}, concentration required to inhibit the cell growth by 50% compared to untreated controls; LC_{50}, concentration which is lethal to 50% of the cells compared to untreated controls; nd, not determined. LC_{50} and IC_{50} values were converted in micromolar (µM) by dividing their values in µg/mL against their respective molecular weight. $SI_{(1)}$ and $SI_{(2)}$ are selectivity indexes against Hs 1. Int and Wl-38 cells, respectively. Compounds with the same letters are not statistically different. Compounds (**1**) globimetulin A, (**2**) globimetulin B, (**3**) globimetulin C and (**4**) 3-O-β-D-glucopyranosyl-α-amyrin were isolated from *Globimetula dinklagei*. Compounds (**5**) 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin and (**6**) apigenin-8-C-β-D-glucopyranoside were isolated from *Phragmanthera capitata*. [a]Significant difference between the compounds tested ($p < 0.05$). [b]Significant difference between the compounds and doxorubicin ($p < 0.05$).

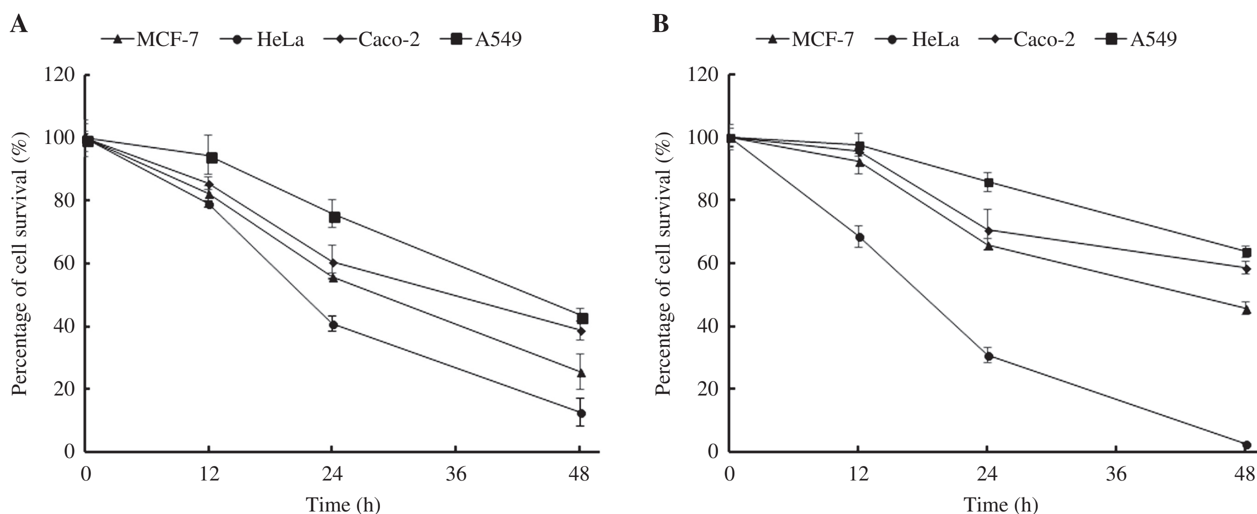

Figure 3: Percentage of cell survival at different times of treatment with the compounds globimetulin B (**2**) (A) and 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**5**) (B) on different cancer cell lines MCF-7, HeLa, Caco-2 and A549. Data are presented as mean ± standard error of the mean of three independent experiments.

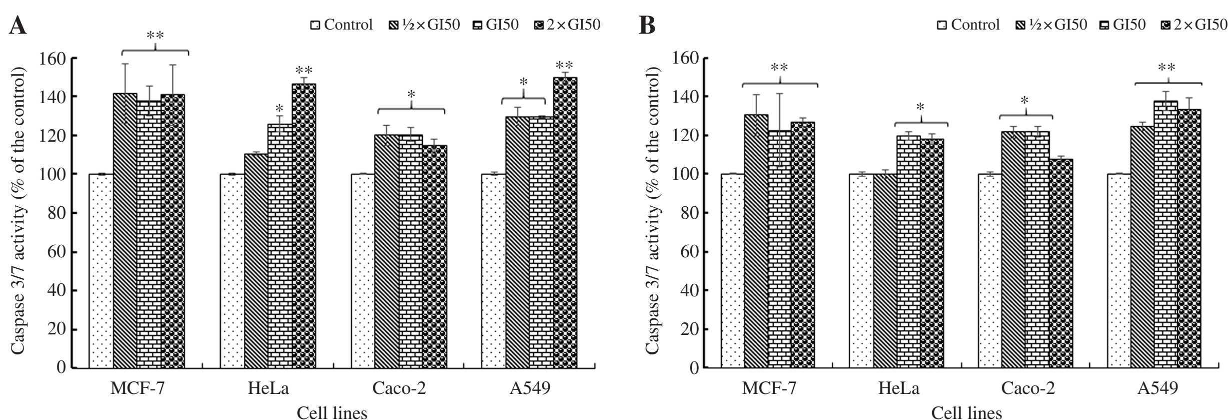

Figure 4: Caspase-3/-7 activation after 24 h of treatment with the compounds globimetulin B (**2**) (A) and 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**5**) (B) on different cancer cell lines MCF-7, HeLa, Caco-2 and A549. The caspase-3/-7 activity is expressed as percentage or fold change to the untreated cells (control). Data are presented as mean ± SD of three independent experiments. $*p < 0.05$ and $**p < 0.01$ indicate the significant difference compared to the control using Dunnett's test.

We observed that the caspase-3 and -7 activity in HeLa cells increased in a concentration-dependent manner when treated with compound **2** (Figure 4A), while compound **5** just induced significant ($p < 0.05$) activation of caspase-3 and -7 (1.2-fold change) when used at concentrations of IC_{50} and $2 \times IC_{50}$ (Figure 4B). The lowest caspase-3 and -7 activation (1.2-fold change) was obtained when using Caco-2 cells although this increase was significant ($p < 0.05$) compared to the untreated cells (control).

4 Discussion

The basis of many cancer treatments is to selectively kill cancer cells with a less toxic effect on normal cells. In the present study, the cytotoxic effect of isolated compounds from *G. dinklagei* and *P. capitata* was evaluated on four cancerous cells and two non-cancerous cells treated with increasing concentrations of these compounds. The difference in the cytotoxicity of compound **2** between the two cell types may be sufficient to allow safe systemic administration without significant side effects as it was observed with the determination of selectivity indexes. However, further comparisons need to be done with other normal cells of specific targeted organs. The low cytotoxic effect of the most potent compound **2** on both non-cancerous cells (from human origin) has confirmed the potential of this compound to be used in future studies to evaluate its mode of action as a chemotherapeutic agent.

SAR is the relation between the chemical structure and pharmacological activity for a series of compounds [19]. This enables the identification of the chemical groups responsible for inducing a target biological effect in the organism. While comparing the cytotoxic effect between triterpenoids, it was found that compound **2** (globimetulin B) exhibited the highest activity, thereby pointing out the fact that the esterification of the hydroxyl group at C-3 has a mainly negative effect on cytotoxicity. This hydroxyl group at C-3 has been reported as an important active group for the cytotoxicity of pentacyclic triterpenoids [20]. The glycosidic linkage at the position C-3 of saponins or triterpenoids has been indicated as having a negative effect on cytotoxicity [20, 21], which may explain the low cytotoxic activity of compound **5** compared to compound **2**. Interesting selective cytotoxicity of some of these compounds was also observed in this study. It has been reported that several triterpenoids and saponins exhibit cytotoxicity against a variety of cancer cells without manifesting any toxicity in normal cells [22, 23]. Compound **5**, which is similar in chemical structure to compound **4** except the presence of a hydroxyl group at C-28, was more potent compared to compound **4**. Both compounds contain a sugar moiety which has been reported to decrease the cytotoxicity of triterpenes closely related to globimetulin and amyrin [24, 25]. It therefore indicates the importance of this free hydroxyl group at C-28 in the cytotoxic effect of compound **5**. However, compound **5** exhibited different cytotoxic activities in different cancer cells types. As an illustration, the IC_{50} values of compound **5** were 4.16 μg/mL (6.88 μM) and greater than 100 μg/mL (>165.56 μM) against HeLa and A549 cells, respectively. The differential cytotoxic effect of this compound (**5**) on these two cancer cells types may depend on the tissue-specific toxicity [26] or the genetic background [27] of these cells that could also influence the choice of this compound in a specific cancer treatment.

Caspases are very important for initiation and execution of apoptosis. Caspases-8 and -9 are known as "initiators" in the apoptotic process, while caspases-3, -6 and -7 are "executioners" [28, 29]. Overall, our results indicate clearly the induction of caspase-3 and -7 activity in treated cells which therefore represent the proof that apoptosis has taken place in the cells. Caspase-3 is known to be involved in the cleavage of many important proteins such as poly(ADP-ribose) polymerase, which leads to the disassembly of cell structure and DNA fragmentation resulting in cell death [30, 31]. Moreover, caspase-7 shares many similarities with caspase-3 since both are executioner caspases and are substrates for initiator caspases in extrinsic or intrinsic apoptotic pathways [28]. However, further studies such as the evaluation of the activity of other caspases (-8, -9), DNA damage, reactive oxygen species content and cell cycle analysis will be performed in a near future to understand the full mechanistic pathway responsible for apoptosis caused by these two active compounds.

5 Conclusion

The aim of this research was to evaluate the selective cytotoxicity of compounds isolated from *G. dinklagei* and *P. capitata* against cancer cells, with an effort to analyze their SARs and to understand the mechanism of action of active compounds. Globimetulin B (**2**) and 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**5**) were more potent on cancerous cells and compound **2** had better selectivity index values than doxorubicin, which is mostly used in the treatment of many cancers. The free hydroxyl group at C-3 of the compounds plays an important role in the cytotoxicity, while the attachment of a glycosylic group at this same position decreases the cytotoxicity. The activation of caspase-3 and -7 in treated cells indicated that cell death may occur via induction of apoptosis. In future studies, the mechanistic pathway involved will be studied. Taken together, this work suggests that compounds **2** and **5** are promising candidates for cancer drug development.

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