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Cytotoxic triterpenes from *Antrodia camphorata* and their mode of action in HT-29 human colon cancer cells

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ABSTRACT

Five lanostane (**2, 3, 4**, **6** and **8**) and three ergostane-type (**1**, **5** and **7**) triterpenes isolated from the fruiting bodies of *Antrodia camphorata* were evaluated for their *in vitro* cytotoxic data against various cancer cell types. The three zhankuic acids, **1**, **5** and **7** displayed the most potent cytotoxic effect with an IC_{50} value of 22.3–75.0 μM. The compound **3** was selectively cytotoxic in three colon cancer cell lines (HT-29, HCT-116 and SW-480) and a breast cancer model (MDA-MB-231), whereas **8** only showed its cytotoxicity against MDA-MB-231. None of these isolates was toxic to mammary epithelial (MCF10A) and primary foreskin fibroblast (HS68) cells, two human normal cell lines. The compounds **1**, **5** and **7** were also demonstrated to induce apoptosis in HT-29 and SW-480 cells, as confirmed by sub-G1 cell cycle arrest. In HT-29 cells, the expression of apoptosis-associated proteins poly-(ADP-ribose) polymerase cleavage, Bcl-2 and procaspase-3 were suppressed by compounds **1**, **5** and **7**. A mixture containing 4 μM each of compounds **1**, **5** and **7** also showed a synergistic cytotoxic effect in HT-29 cells.

1. Introduction

Cancer is a major cause of death worldwide and causes serious problems in human life, including mental and physical agony and economic strain. Therefore, many kinds of cancer therapies, including various anticancer agents, have been developed. However, they also have several problems such as serious side effects and drug resistance [1]. To resolve these difficulties, development of cancer chemopreventive agents and improvement of cancer treatment are very important. Accordingly, screening of natural products as potential anticancer agents, in the form of functional foods or nutraceuticals has become an important undertaking [2]. The rising interest in the pharmacological properties of natural triterpenoids [3] led us investigate *in vitro* cytotoxicities of five lanostane (**2, 3, 4**, **6** and **8**) and three ergostane-type (**1**, **5** and **7**) triterpenes isolated from *Antrodia camphorata* (Polyporaceae).

A. camphorata is a parasitic fungus grown on the hardwood of *Cinnamomum kanehirai* Hay (Lauraceae), has been widely used as a Chinese remedy for food and drug intoxication, diarrhea, abdominal pain, hypertension and cancer [4]. Crude extracts from the fruiting bodies and mycelium of *A. camphorata* show potent anticancer activities in human leukemia HL-60, breast cancer MDA-MB-231 (estrogen-nonresponsive) and MCF-7 (estrogen-responsive)

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cells, but not in healthy breast cells (HBL100) and umbilical vein endothelial cells [5–7]. The extracts from fruiting bodies show cytotoxic effect in bladder cancer cells and arrest the cell cycle in the G2/M phase [8]. The ethanol extract from mycelium inhibits the proliferation of human lung cancer cell A-549, but not the normal fetal lung fibroblast MRC-5 cell [9]. The methanol extract of mycelium inhibits cell viability and induces apoptosis in Hep G2 via G0/G1 cell cycle arrest followed by the activation of the caspase-3 and -8 cascades [10]. The ethyl acetate extract (EAC) inhibits cell growth in two liver cancer cells, Hep G2 and PLC/PRF/5 through the regulation of Bcl-2 family protein expression [11]. *A. camphorata* treatment could be effective in inhibiting breast cancer cell MDA-MB-231 proliferation and inducing apoptosis *in vitro* and *in vivo* [12]. Interestingly, the authors also show the nontoxicity of *A. camphorata* with a daily oral administration of 500 mg/kg for 28 days in rats, which increase its potential for application in food and drug products [13]. Hence, it is of significant interest to isolate and identify such exact compounds which are responsible for the anticancer activity of this fungus.

As part of a study program to evaluate the therapeutic properties of *A. camphorata*, this paper has demonstrated the *in vitro* cytotoxic effects of eight triterpenoids (Fig. 1) against various cancer types: colon, liver, breast and lung cancer cell lines. Although the *in vitro* cytotoxic activity of zhankuic acids A (**5**) and C (**7**) in P-388 murine leukemia cells had been reported [14], however no information about their mechanism of action. In this study, among eight selected compounds, three ergostane-types were capable of blocking cell cycle progression at the sub-G1 phase and inducing apoptosis through the cleavage of the downstream poly(ADP-ribose) polymerase, pro-caspase-3 and Bcl-2. In addition, the combination of ergostane-triterpenes exhibited a potential synergistic cytotoxic effect in HT-29 cells.

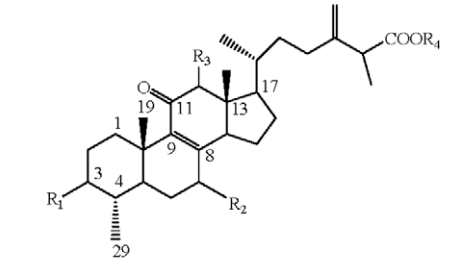
2. Materials and methods

2.1. Materials

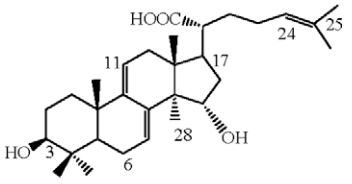
All the materials were obtained commercially and used without further purification. NMR spectra were measured on a Varian Unity Inova-600 VXR-300/51 spectrometer with TMS as an internal standard. Silica gel for column chromatography (CC) (0.063–0.200 mm), was a product of Merck Company. TLC was performed on Merck TLC plates (0.23 mm thickness), with compounds visualized by spraying with 8% (v/v) H_2SO_4 in ethanol and then heating on a hot plate. The wild fruiting bodies of *A. camphorata* were collected from the Yuli, Hualien County, Taiwan, in December 2006. A voucher specimen (YMT 6002) was deposited in the Herbarium of the Institute of Biochemical Sciences and Technology, Chaoyang University of Technology, Taiwan, ROC.

2.2. Extraction and isolation

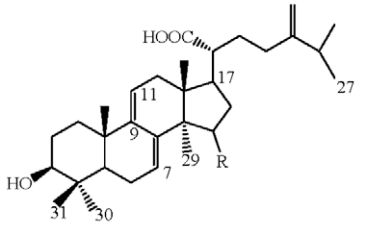
The compounds **1**–**8** were separated from the fruiting bodies of *A. camphorata*, according to the extraction and isolation procedures described by Male et al. [15]. Briefly, air-dried powder of *A. camphorata* was extracted with $CHCl_3$ using a soxhlet extractor. After solvent evaporation, the residue subjected to silica gel column chromatography was eluted with increasing polarity using mixtures of *n*-hexane/EtOAc. Following the TLC analysis, eluates of



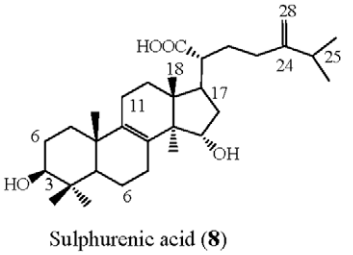
Methyl antcinate B (**1**): R_1=R_2=(=O), R_3=H, R_4=CH_3
Zhankuic acid A (**5**): R_1=R_2=(=O), R_3=R_4=H
Zhankuic acid C (**7**): R_1=R_3=(α-OH), R_2=(=O), R_4=H



3β, 15α-dihydroxy lanosta-7,9 (11), 24-triene-21-oic acid (**4**)



Dehydroeburicoic acid (**2**): R=H
15α-Acetyl-dehydrosulphurenic acid (**3**): R= (α-OAc)
Dehydrosulphurenic acid (**6**): R=(α-OH)



Sulphurenic acid (**8**)

Fig. 1. Chemical structures of the eight isolates from *Antrodia camphorata*.

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similar profiles were combined to give five fractions (A–E). The fraction B was purified by CC to afford methyl antcinate B or methyl zhankuic acid A (**1**), dehydroeburicoic acid (**2**) and 15α-acetyl dehydrosulphurenic acid (**3**), respectively. From the fraction C, compounds 3β,15α-dihydroxy lanosta-7,9 (11), 24-triene-21-oic acid (**4**) and zhankuic acid A (**5**) were obtained. The fraction D was further separated using a silica gel column eluting with a gradient of *n*-hexane/EtOAc, to afford five subfractions. Dehydrosulphurenic acid (**6**) and zhankuic acid C (**7**) were obtained from subfractions D–3 and D–4, respectively. Sulphurenic acid (**8**) was obtained from the fraction E. The structures of compounds **1–8** were determined by ^{1}H and ^{13}C NMR spectroscopy and by comparison of the spectral data with those published values [14–17].

2.3. Sulforhodamine B assay

Sulforhodamine B (SRB) (Sigma–Aldrich Chemie GmbH, Munich, Germany) was used to test the effects of active compounds on cell growth and viability. Based on the method described by Vichai and Kirtikara [18], compounds **1–8** were dissolved in dimethylsulfoxide (DMSO) before diluting with the growth medium to a final DMSO concentration of <0.05%. The cancer cells were seeded into 96 well plates in the growth medium at 3000 cells/well. After 24 h of incubation, the medium was replaced with a fresh growth medium containing the test compounds **1–8** (0, 25, 50, 100 and 200 μM). The cells were incubated for another 48 h. The cells were fixed with TCA by gently adding 50 μl TCA (50%) to each well to a final TCA concentration of 10% with subsequent incubation for 1 h at 4 °C. The plates were then washed 5 times with deionized water and air dried. The dried plates were stained with 100 μl of 0.4% (w/v) SRB prepared in 1% (v/v) acetic acid for 10 min at room temperature. The plates were rinsed quickly four times with 1% acetic acid to remove unbound dye, followed by air-drying until no moisture was visible. The bound dye was solubilized in 2 mM Tris base (100 μl/well) for 5 min on a shaker. Optical densities were read on a microplate reader (Molecular Devices, Sunnyvale, CA) at 562 nm.

2.4. Annexin V/propidium iodide (PI) assay

Apoptosis was assessed by evaluation of the annexin V binding to phosphatidylserine (PS) that was externalized early in the process of apoptosis. The annexin V binding to cells was determined using a commercially-available annexin V apoptosis detection kit (R&D Systems, Minneapolis, MN) and flow cytometry. Following the treatment of the colon cancer cells (1×10^6 cells/ml) with different doses of compounds **1, 5** and **7** for 48 h, resulting cells were recovered and washed twice with 2 ml of ice-cold phosphate-buffered saline (PBS). Cells were incubated with 100 μl of HEPES buffer containing 2 μl of fluorescein isothiocyanate (FITC)-conjugated annexin V and 2 μl of propidium iodide (PI) for 15 min. Following the incubation, without washing the cells of excess reagents, 400 μl binding buffer was added. The stained cells were immediately analyzed with a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA). Approximately 10,000 counts were acquired for each sample. The percentage distribution of apoptotic cells was calculated by CellQuest™ software (Becton and Dickinson).

2.5. Cell cycle analysis

Propidium iodide (PI) staining and flow cytometry were used to determine the cell cycle stage. Cells were treated with different doses of compounds **1–8** for 48 h at 37 °C, and the control cells were treated with the normal medium. The 2×10^6 treated cells were washed with 5 ml of PBS, and trypsinized at 37 °C for 5–10 min. Cells were spun down and washed with 5 ml of PBS. Cells were resuspended in 500 μl of PBS and fixed with 4.5 ml of 70% ethanol with gentle vortexing. Cells were allowed to settle overnight at −20 °C. Fixed cells were spun down and washed with 5 ml PBS. The cells were resuspended in 500 μl of PI (2 μg/ml)/Triton X-100 (0.1% v/v) staining solution with RNase A (200 μg/ml) in the dark, and analyzed by a flow cytometer. The staining solution was purchased from Chemicon International (Temecula, CA).

2.6. Western blot analysis

Compounds **1–8**-treated and untreated cells were rinsed twice with PBS (pH 7.0) and the total proteins were extracted by adding 200 ml of a cold lysis buffer to the cell pellets on ice for 30 min, followed by centrifugation at 10,000*g* for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured by the Bradford assay with bovine serum albumin (BSA) as the standard. The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 mM Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol and 0.1% bromophenol blue. After boiling at 95 °C for 5 min, the mixture was subjected to 12% SDS–polyacrylamide minigels at a constant current of 20 mA. Electrophoresis was carried out on SDS–polyacrylamide gels (SDS–PAGE). Following electrophoresis, proteins on the gel were electrotransferred onto an Immobilon membrane (PVDF, Millipore®) with a transfer buffer consisting of 25 mM Tris–HCl (pH 8.9), 192 mM glycine and 20% methanol. The membrane was washed with Tris-buffered saline (TBS) (10 mM Tris and 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% nonfat dried milk. The membrane was further incubated overnight at 4 °C with respective specific antibodies, such as caspase-3 (1:1000), PARP (1:1000), Bcl-2 (1:1000) and β-actin (1:5000). After hybridization with primary antibodies, the membrane was washed with TBST three times. The membrane was then incubated with a horseradish peroxidase-labeled secondary antibody for 45 min at room temperature, and washed with TBST three times. The final detection was performed with enhanced chemiluminescence (ECLTM) Western blot reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.7. Statistical analysis

Each experiment was performed in triplicate and repeated three times ($n = 9$). The results were expressed as

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means ± SD. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Cell proliferation

To investigate the cytotoxic potential of the eight isolates (Fig. 1), ten cancer and two normal cell lines were incubated with each isolate with increasing concentrations for 48 h. The effects on cell proliferation were determined by the sulforhodamine B assay [18]. The three ergostane-type triterpenes methyl antcinate B (**1**), zhankuic acids A (**5**) and C (**7**) showed a wide range of potency with IC_{50} values ranging from 22.3 to 75.0 μM in various cancer cell types (Table 1). Three isolates exhibited similar activities in the cell lines in which they were examined, with compound **1** being slightly more potent than **5** and **7**. The highest potency (IC_{50} ~ and/or <30 μM) was observed against the human colon cancer cell lines HT-29 and SW-480, the liver cell lines Huh-7 and Hep G2, the breast cell line MDA-MB-231, and the lung cell line A-549 (Table 1). As estimated by the ACD/Structure Designer software (Advanced Chemistry Development, Toronto, ON, Canada), the aqueous solubility of compound **1** at pH 7 is only 2×10^{-4} mg/ml compared to 0.11 mg/ml and 0.24 mg/ml for **5** and **7**, respectively. Compound **1** is neutral with no p*Ka* whereas **5** has one apparent p*Ka* value: 4.59 (position 11) and **7** have three p*Ka* values: 14.83 (position 3), 12.33 (position 11) and 4.59 (position 12). Apparently, hydrophobicity has played an important role in cytotoxic data of this zhankuic acid family.

Compounds **2, 3, 4**, **6** and **8** responded differentially to different tumor types. Compound **3** was more cytotoxic for the colon cancer cell lines with an IC_{50} value of 59.8, 79.9 and 86.5 μM against HT-29, HCT-116 and SW-480, respectively, compared to the lung cancer cell lines A-549 and CL1-0 (IC_{50} > 500 μM, Table 1). Similar behavior was observed for sulphurenic acid (**8**) with an IC_{50} value of 89.2 μM against the breast cancer

Table 1
Cytotoxicity of *A. camphorata* triterpenes **1–8** on human tumor and normal cell lines as calculated from dose response curves.[a]

Cell type	Cell line	Compounds IC_{50}[b] (μM)[c]							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Tumor									
Colon	HT-29	22.3	387.0	59.8	233.0	30.0	MR[d]	28.0	293.0
	HCT116	32.1	336.0	79.9	274.0	38.3	MR	42.6	289.0
	SW480	27.3	MR	86.5	276	30.7	MR	44.0	254.0
Liver	Huh7	30.4	MR	134.0	MR	42.8	MR	45.1	428.0
	HepG2	23.4	MR	MR	MR	38.6	MR	48.7	171.0
	Hep3B	68.3	MR	326.0	MR	48.4	MR	50.7	476.0
Breast	MDAMB231	25.1	370.0	55.9	133.0	25.6	339.0	27.8	89.2
	MCF-7	57.8	MR	MR	379.0	36.1	MR	53.0	357.0
Lung	A549	25.1	129.0	MR	312.0	41.9	194.0	34.4	MR
	CL1-0	31.5	140.0	MR	476	75.0	241.0	64.3	MR
Normal									
Mammary epithelial	MCF10A	413	MR	MR	MR	473	MR	MR	MR
Foreskin fibroblast	HS68	MR	MR	MR	MR	MR	MR	MR	MR

[a] Sigmoidal dose response curves (variable slopes) were generated using GraphPad Prism V. 4.02 (GraphPad Software Inc.).
[b] Tested compound concentration required to inhibit cell proliferation by 50% after 48 h of treatment compared with vehicle control DMSO.
[c] Values are the mean of triplicate of at least two independent experiments.
[d] MR = IC_{50} value > 500 μM.

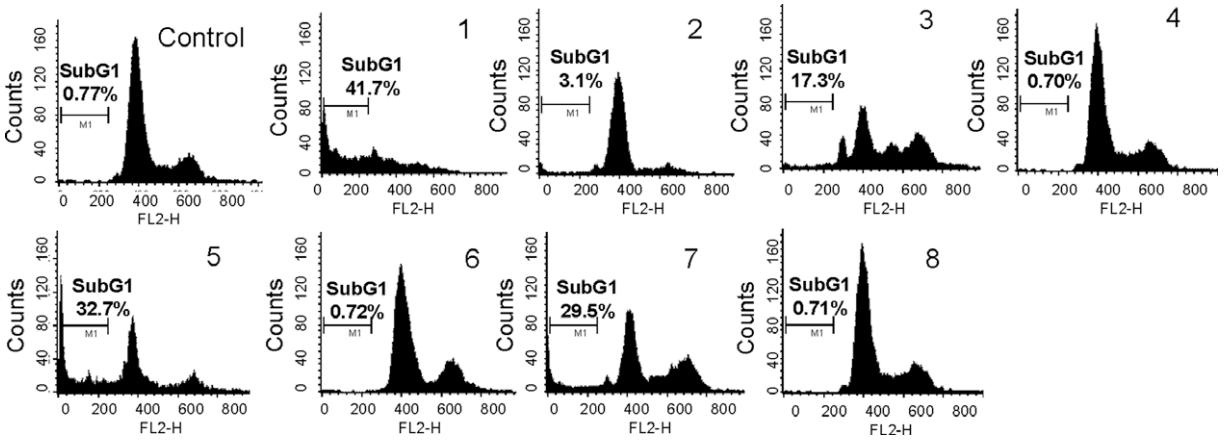

Fig. 2. Detection of $SubG_1$ accumulation in HT29 cells after 48 h incubation with compounds **1–8**. Cells were cultured in the absence (control) or presence of 50 μM of compounds **1–8** for 48 h. The harvested cells were stained with propidium iodium and DNA content analyzed by flow cytometry. The percentage of apoptotic cells was assessed by counting the cells with DNA contents below 2 N.

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cell line MDA-MB-231 (Table 1). The isolates **2**, **3**, **4**, **6** and **8** exhibited no significant activity (IC_{50} > 500 μM) against liver, lung, liver, colon, liver and lung tumor cell lines (Table 1).

Further, the subject active compounds exhibited the variable growth inhibition against different cancer cell lines of the same tumor type. The compound **1** exhibited a strong growth inhibition (IC_{50} = 23.4 μM) against the liver cancer cell line Hep G2, whereas it only showed a moderate effect in Hep 3B (IC_{50} = 68.3 μM). The same phenomenon was observed by compounds **1** and **8** in the breast cancer cell lines MDA-MB-231 and MCF-7 with an IC_{50} value of 25.1 and 89.2; and 57.8 and 357.0 μM, respectively (Table 1). Collectively, the data in Table 1 clearly shows that the cytotoxic effects of compound **1** are similar in all the cancer cell lines tested, while compound **5** is not so effective in lung and liver cell lines and compound **7** is more effective on colon cancer cell lines. Interestingly, the potent cytotoxic compounds **1**, **3**, **5**, **7** and **8** were less sensitive (i.e., IC_{50} values ranging from 413 to >500 μM) to the inhibitory activity of two human normal cell lines: mammary epithelial (MCF10A) and primary foreskin fibroblast (HS68) cells (Table 1). These results pointed to the differential (selective) effect between tumor and normal cells of the afore-mentioned compounds.

Although it was somewhat difficult to elucidate an appropriate structure versus activity relationship, the experimental data revealed that the presence of ketone groups and a double bond position were important for the activity. Compounds **2** differs from **1** and **5** in that there are no ketone groups at positions 3, 7 and 11, while the position 3 contains a hydroxyl group and the carboxylic acid group is at the position 21 rather than 26. The removal of the three ketone groups drastically reduced the inhibitory effect as the IC_{50} was determined to be ranging from 129 to >500 μM (Table 1). The remaining four compounds **3**, **4**, **6** and **8** are similar to **2**, except for some small structural differences. The compounds **4** and **6** only differ with respect to a double bond at the position 24, where the former shows higher inhibitory potency against colon cancer cells than the latter. The compound **8** is selectively cytotoxic in MDA-MB-231, and the only one that does not contain the diene structure within the rings at positions 7 and 9 and instead possesses a single double bond in the rings at the position 8. The compound **3** showed higher inhibitory effect in colon cancer cells, compared to **6**, and such an effect could be attributed to the addition of the bulky acetoxy group at the position 15 of **3**. The cytotoxic results of **1**, **5** and **7** are in parallel with a previous report on *Spodoptera frugiperda* *Sf*9 insect cells [15], and **5** in P-338 murine leukemia cells [14]. To our knowledge, we have demonstrated here for the first time the cytotoxicity of *A. camphorata* isolates on a wide range of cancer cell types.

3.2. Cell cycle arrest and apoptosis in human colon cancer cells

To investigate whether the observed cytotoxic effects are caused by apoptosis, HT-29 cells were treated with or without compounds **1–8**; and resulting cell cycles (sub-G1 group) were examined by flow cytometry. Compounds **1, 5** and **7** elevated the percentage of HT-29 cells in the sub-G1 phase from the control value of 0.77% to 41.7%, 32.7% and 29.5%, respectively, at 50 μM for 48 h incubation while the other isolates had no effect (Fig. 2). These observations are consistent with the hypoth-

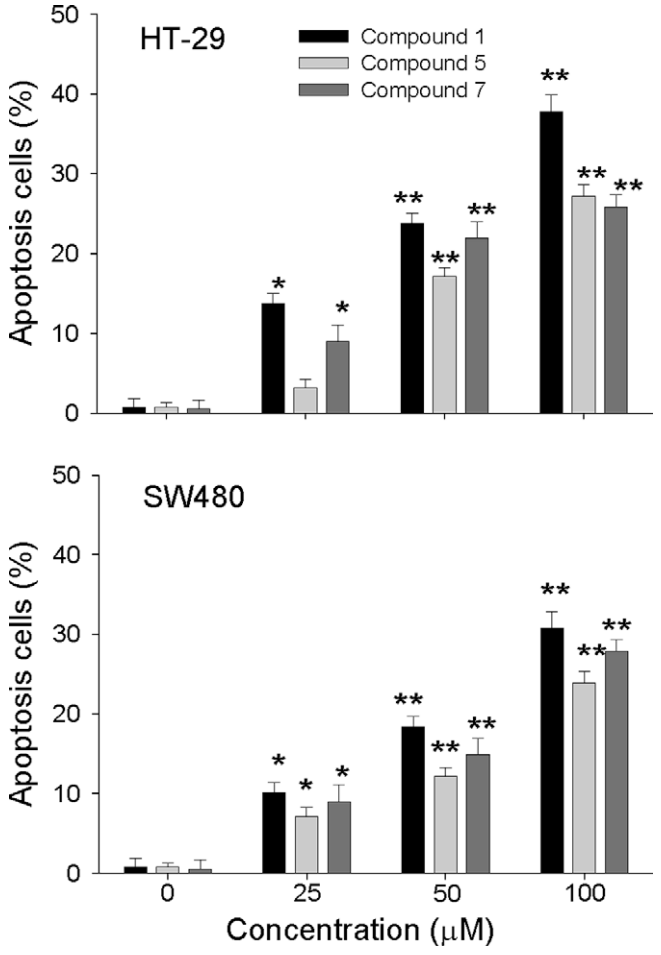

Fig. 3. Compounds **1, 5** and **7** promote apoptosis in the human colon cancer cells. HT29 and SW480 cells were treated with a range of **1, 5** and **7** (0, 25, 50 and 100 μM) for 48 h, and the cells were stained with annexin V/PI and analyzed by flow cytometry. Bar graphs represent the mean values of triplicate determinations ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with the control.

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esis that the active isolates would kill human cancer cell lines in a mechanism-specific manner [19]. Furthermore, the compounds **1, 5** and **7** (i.e., IC_{50} < 30 μM) determined from the initial cell line screen (Table 1) were subsequently tested in the concentration range 25–100 μM against colon cancer cells HT-29 and SW480; and apoptosis was assessed by evaluation of the annexin V binding to phosphatidylserine. The results revealed that compounds **1**, **5** and **7** were able to induce apoptosis in a concentration-dependent manner after 48 h of incubation (Fig. 3), which demonstrated the same pattern as that observed in the cytotoxic measurement (Table 1). Among the three compounds, **1** being slightly potent, promoted the apoptosis in the HT-29 and SW480 cells by 37.76% and 30.70%, respectively, at the concentration of 100 μM whereas **5** and **7** displayed such an effect in a range from 23.80% to 27.80%.

3.3. Effect on expressions of poly-(ADP-ribose) polymerase (PARP), Bcl-2 and pro-caspase-3 in HT-29 cells

Apoptosis is characterized as an ordered process in which multiple events take place including post-translation modifications of key regulatory molecules such as protein kinases, Bcl-2 family members and caspases [20,21]. Since triterpene derivatives could trigger apoptosis and induce cell cycle arrest [22,23], a series of experiments was conducted to examine the expressions of apoptosis-associated proteins in HT-29 cells in the presence or absence of **1–8** by the Western blot analysis. Despite the differences in the structure of the isolates and their cytotoxic efficacy (Table 1), PARP cleavage was elevated by treatment with compounds **1, 5** and **7** (Fig. 4). More specifically, nuclear full length PARP was decreased, and the cleaved form of PARP in the nuclei was significantly increased after 48 h of treatment (Fig. 4). Similar results were obtained for the inhibition of Bcl-2 and pro-caspase-3 expression. During the apoptosis, PARP has been reported to be proteolyzed directly by active caspase-3, resulting in the inactivation of PARP for DNA repair [20,21]. Our results were consistent with these findings and demonstrated that compounds **1**, **5** and **7** induced apoptosis in HT-29 cells (Fig. 3), which were mediated through the mitochondrial dependent pathway.

3.4. Combination treatment on growth inhibition of HT-29 cells

The cytotoxic activity of compounds **1**, **5** and **7** alone (6 and 12 μM) or in combination (4 and 6 μM) were also examined in the colon cancer model HT-29 cell by the SRB assay. This model was chosen due to the potent *in vitro* sensitivity of this cell line to these compounds (IC_{50} = 22.3, 30.0 and 28.0 μM for **1**, **5** and **7**, respectively, Table 1). As shown in Fig. 5, the maximal inhibition was attained with a reduced dosage of each of the individual compound. In the combination of 6 μM of compounds

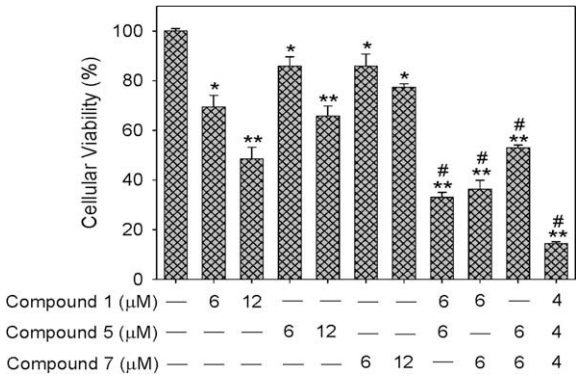

Fig. 5. The synergistic effect of two or three compounds combined in HT-29 cells growth inhibition. The inhibitory effect of compounds **1**, **5** and **7** on HT-29 cellular viability was measured by the SRB assay. The data are expressed as the mean ± SD of three experiments ($^{*}p < 0.05$; $^{**}p < 0.01$ compared with the control) ($^{\#}p < 0.01$ for combination compared with either one single agent).

1 + **5**, **1** + **7** and **5** + **7**, the HT-29 cell viability was inhibited by 67.1%, 63.7% and 47.0%, respectively. In particular, the combination of 4 μM of **1** + **5** + **7** exhibited a remarkable effect on cell viability, decreasing by 85.6%. Such a result suggested a significant synergistic effect among the three compounds.

In conclusion, this study has demonstrated that triterpenes from *A. camphorata* inhibited cell proliferation in colon, liver, breast and lung cancer cell lines. These isolates also blocked the cell cycle progression at the sub-G1 phase; and induced apoptosis in colon cancer cells through the cleavage of the downstream poly(ADP-ribose) polymerase, pro-caspase-3 and Bcl-2. In addition, the combination of ergostane-triterpenes exhibited a potent synergistic cytotoxic effect in HT-29 cells. Further studies are needed to establish the *in vivo* anti-tumor activity and elucidate the underlying mechanism of action.

4. Conflicts of interest

None declared.

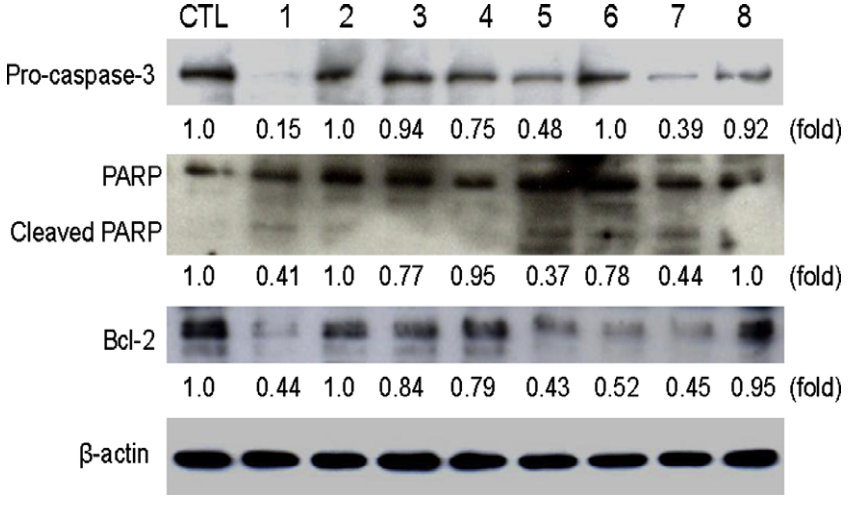

Fig. 4. Western blot analysis for pro-caspase-3, PARP and Bcl-2 protein expression in HT-29 cells treated with compounds **1–8**. Cells were treated with 50 μM of each compound for 48 h. Lanes 1–8 are the results for compounds **1–8**, respectively. Inhibition of pro-caspase-3, PARP and Bcl-2 protein expression could be seen for compounds **1, 5** and **7**, with **1** showing the highest inhibition. Relative expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-actin reference bands.

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