

Original Article

Plaunotol from *Croton stellatopilosus* Ohba inhibited cell growth and induced apoptosis in human cancer cell lines

Charoenwong Premprasert, Supreeya Yoenyongsawad, Supinya Tewtrakul, and Juraithip Wungsintaweekul*

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand

Received: 6 November 2017; Revised: 5 March 2018; Accepted: 10 April 2018

Abstract

Plaunotol is an acyclic diterpene alcohol that was evaluated for anti-proliferative activity in four human cancer cell lines: HeLa; HT-29; MCF-7; and KB. After the cells were treated with plaunotol, cell viability was determined using the MTT assay. The results showed that plaunotol inhibited the growth of human cancer cell lines with an IC_{50} of $65.47 \pm 6.39 \mu\text{M}$ (HeLa), $72.92 \pm 5.73 \mu\text{M}$ (HT-29), $80.90 \pm 3.48 \mu\text{M}$ (KB), and $62.25 \pm 9.15 \mu\text{M}$ (MCF-7). For apoptotic detection, the cells treated with plaunotol were stained with Annexin-V/7-AAD reagent. The results indicated that plaunotol induced cell death or apoptosis. The transcription profile of apoptotic-associated genes, including *TNF- α* , *BCL-2*, *BAX*, and *BAK* genes, was determined using the qRT-PCR technique and the expression level was calculated as the relative quantitation (RQ). The ratio between RQ of *BCL-2* and *BAX* suggested that plaunotol significantly induced apoptosis in HeLa, MCF-7, and HT-29 cell lines, in particular.

Keywords: plaunotol, anti-proliferative activity, MTT assay, apoptosis, human cancer cell lines

1. Introduction

Plaunotol is an acyclic diterpene alcohol derived from geranylgeranyl diphosphate (Figure 1). Plaunotol was first isolated in 1978 and was found only in *Croton stellatopilosus* Ohba or plaunoi (Thai name) (Ogiso *et al.*, 1978). Plaunotol was claimed to have anti-peptic ulcer ability and it was successfully registered with the World Health Organization (WHO) (Ogiso *et al.*, 1985). It was further processed into a soft gelatin capsule under the trade name Kelnac[®] by Sankyo Daiichi, Japan. Recently, the partially purified plaunotol extract was reported to be potentially safe after acute and chronic oral toxicity tests in an animal model (Chaotham, Chivapat, Chaikitwattana, & De-Eknamkul, 2013).

Plaunotol has a gastro-protective effect that mediates several mechanisms such as releasing prostaglandins (PGE₂ and PGI₂) and secretin as well as inhibiting neutrophil

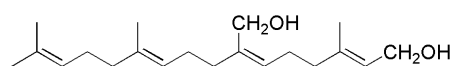


Figure 1. Chemical structure of plaunotol.

activation (Murakami *et al.*, 1999; Shiratori, Watanabe, & Takeuchi, 1993; Ushiyama *et al.*, 1987). Considering *Croton* species, some diterpenoids were reported to have anticancer and antitumor activities such as *trans*-dehydrocrotonin and *trans*-crotonin from *C. cajucara* (Grynberg *et al.*, 1999), neocrotocembranal from the stem bark of *C. oblongifolius* (Roengsumran *et al.*, 1999), and *ent*-15-oxo-kaur-16-en-18-oic acid from the bark of *C. argyrophyloides* (Santos *et al.*, 2009). Nowadays, several secondary metabolites with interesting biological activities serve as leading compounds in the development of drugs such as paclitaxel and docetaxel. Both drugs are strong anticancer agents and are already being used in the treatment of cancer. Interestingly, other isoprenyl molecules such as farnesol (Joo, Liao, Collins, Grissom, & Jetten, 2007; Park *et al.*, 2014), geranylgeraniol (GGOH) (Ohizumi *et al.*, 1995; Yoshikawa *et al.*, 2009), geranylgeranoic (Nakamura *et al.*, 1996; Shidoji *et al.*, 2006; Shidoji,

*Corresponding author

Email address: juraithip.w@psu.ac.th

Nakamura, Moriwaki, & Muto, 1997), and geranylgeranylacetone (Jo *et al.*, 2016; Yoshikawa *et al.*, 2010) were reported to inhibit cancer cell growth via apoptosis induction.

Plaunotol was identified as a cytoprotective anti-peptic ulcer agent. More recently, plaunotol was reported to possess antitumor activity against gastric cancer cell lines (MKN-45, MKN-74, and AZ-521) by apoptosis. It inhibited the growth of gastric cancer cells and induced caspase activation including caspase-3, -8, and -9 (Yamada *et al.*, 2007). Moreover, plaunotol was reported to exhibit a cytotoxic effect against the DLD1 human colon cancer cell line (Yoshikawa *et al.*, 2009).

Since the discovery of signaling pathways in human cancer cells, these pathways have shown to be connected with a network of regulation at the molecular level of cell growth and cell division. Apoptosis is a biological process in multicellular organisms that plays an important role in animal development and homeostasis (Fuchs & Steller, 2011). During apoptosis, the cell is initially defined by its biochemical characteristic changes, including cell shrinkage, blebbing of the plasma membrane, and nuclear morphology (which includes the chromatin condensation and fragmentation, and the release of apoptotic mediators) (Prayong, Weerapreeyakul, & Barusrux, 2007). The cell cycle is regulated by a complex sequence of signaling pathways by which a cell grows, duplicates its DNA, and divides (Cooper, 2000). Nevertheless, in cancer cells, the cell cycle process fails and results in an uncontrolled cell proliferation. For this reason, a better understanding of cancer biology and cancer genetics is necessary for cancer research. Many research reports focused on the anti-proliferative effects on cancer cells, its mechanisms, and the realization that the cell cycle progression, apoptosis, and regulatory genes involve apoptosis and the cell cycle.

Based on the above rationale, plaunotol may exert anticancer effects in human cancer cell lines. Therefore, the present study was undertaken to assess the effect of plaunotol on the growth of cancer cells and also its mechanism was investigated via apoptosis. The study reported on an investigation of the molecular mechanism of plaunotol on the anti-proliferative effect, induction of apoptosis, and accumulation of cells in cell cycle phase. In addition, a transcription analysis of the associated-apoptotic mediators on four cancer cell lines that included HeLa, HT-29, MCF-7, and KB cells was also investigated.

2. Materials and Methods

2.1 Isolation of plaunotol

Plaunotol was extracted from *C. stellatopilosus* leaves using silica gel column chromatography described by Premprasert *et al.* (2013). The structure was confirmed by spectroscopic methods [¹H-, ¹³C-NMR and MS].

Plaunotol: a pale yellow oil; IR ν_{\max} cm^{-1} 3300 (O-H), 1665 (C=C), 1440 (C-H), 1380, 1000; ¹H NMR (CDCl₃, 500 MHz) δ : 1.60 (3H, s, H-19), 1.60 (3H, s, H-20), 1.64 (3H, s, H-16), 1.64 (3H, s, H-17), 1.95 (2H, m, H-13), 2.02 (2H, m, H-12), 2.02 (2H, m, H-4), 2.10 (2H, m, H-8), 2.16 (2H, m, H-5), 4.05 (2H, s, H-18), 4.07 (2H, d, $J = 7.1$ Hz, H-1), 5.06 (1H, m, H-10), 5.09 (1H, m, H-14), 5.22 (1H, m, H-6); ¹³C NMR (CDCl₃, 125 MHz) δ : 123.9 (C-10), 124.0 (C-14), 124.2 (C-

2), 127.4 (C-6), 131.3 (C-11), 135.3 (C-15), 138.8 (C-7), 138.9 (C-3), 15.9 (C-20), 16.4 (C-17), 17.6 (C-19), 25.6 (C-16), 25.8 (C-5), 26.6 (C-13), 26.7 (C-9), 34.8 (C-8), 39.2 (C-4), 39.6 (C-12), 58.9 (C-18), 59.8 (C-1); EI-MS at m/z 306.255 (M⁺).

2.2 Chemicals

All chemicals used in this study were of analytical grade. Dulbecco's Modified Eagles's Medium (DMEM), trypan blue, trypsin-EDTA, fetal bovine serum (FBS), and MTT; 3-(4, 5-dimethyl-2-thiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide were from Gibco® BRL, California, USA. The antibiotics (penicillin G [100 U/mL] plus streptomycin [100 $\mu\text{g/mL}$]) were supplied by Invitrogen®, California, USA. The Total RNA mini kit used for total RNA isolation was from Geneaid®, New Taipei City, Taiwan. TaKaRa One Step SYBR® PrimeScript™ RT-PCR Kit II was purchased from Takara Bio Inc., Japan. Primers used for qRT-PCR were designed from the Gene Bank information of *Homo sapiens* (<http://ncbi.nlm.nih.gov>). Assay kits; Muse™ Annexin-V et Dead cell reagent and Muse™ cell cycle reagents were purchased from Merck, Darmstadt, Germany.

2.3 Cell lines

Cells, including human breast carcinoma cell line (MCF-7; CLS No. 300273), human cervical carcinoma cell line (KB, CLS No. 300446), human cervix adenocarcinoma (HeLa, CLS No. 300194), and human colon adenocarcinoma (HT-29, CLS No. 300215) were obtained from the Cell Line Service, Heidelberg, Germany. The human gingival fibroblast (HGF) cell line was kindly provided by the Faculty of Dentistry, Prince of Songkla University. Cells were maintained in DMEM supplemented with 10% FBS and 2% antibiotics (penicillin and streptomycin) at 37 °C in a humidified atmosphere containing 5% CO₂. The monolayered cells were sub-cultured weekly with 0.25% trypsin-EDTA until they reached 80% confluence.

2.4 Cell viability assay

Cell viability was evaluated by the MTT assay with some modifications (Mosmann, 1983). The cells were washed twice with PBS briefly then harvested using trypsin-EDTA. The harvested cells (1×10^3 cells per well) were each placed in a well of a 96-well plate. These cells were attached to the plate after 24-h incubation at 37 °C in an atmosphere of 5% CO₂. Aliquots of medium containing different concentrations of plaunotol (3, 10, 30, and 100 μM) were added and the cell cultures were incubated for 48 h. This experiment established that the use of DMSO concentrations (0.2 % v/v) in the cell cultures caused no cell damage. After the incubation period, the culture medium was removed and washed twice with PBS. Then 100 μL of MTT reagent (5 mg/mL in PBS) was added to each well and the cells were incubated for 3 h. Cell viability was assessed by the ability of metabolically active cells to reduce tetrazolium salt to formazan crystal. The obtained formazan crystals were dissolved in acidic condition (100 μL of 0.01 N HCl in isopropyl alcohol). The absorbance of the samples was analyzed with a microplate reader (DTX 880 multimode detector, Beckman Coulter Inc, Austria) using a

test wavelength of 570 nm. The correlation between concentration and % inhibition was interpreted at 50% inhibitory concentration (IC₅₀) values in micromolar units.

2.5 Apoptosis detection

To evaluate the apoptotic activity of plaunotol, Annexin-V and 7-AAD double staining was carried out as described by Vermes, Haanen, Steffens-Nakken, H. & Reutelingsperger (1995). The human cancer cell lines, i.e. HeLa, HT-29, KB, and MCF-7, were separately seeded briefly at 5×10^5 cells per well and treated with 75 μ M and 150 μ M of plaunotol for 48 h. Paclitaxel at a concentration of 1 μ M was used as the positive control. After treatment, the cells were washed twice with PBS and harvested with 0.25% trypsin-EDTA, followed by centrifugation at 500g for 5 min. Then, the cell pellet was re-suspended in 100 μ L of DMEM media containing 1% FBS followed by the addition of 100 μ L of Muse™ Annexin V et Dead cell reagent. The solution was incubated for 20 min at room temperature under dark conditions. Finally, the stained cells were analyzed by flow cytometer using a Muse™ cell analyzer. The data was analyzed using Muse™ 1.4 software and the data were shown as a four-quadrant dot plot. The statistics on four cell populations were obtained and the populations in each quadrant predicted the apoptosis in live, early apoptosis, late apoptosis, and death, respectively.

2.6 Cell cycle analysis

HeLa, HT-29, KB, and MCF-7 cancer cells were seeded into a 6-well plate at 5×10^5 cells/well. The cells were then treated with various doses of plaunotol (25 μ M to 100 μ M) and with DMSO (0.2%) as a control group. The treated cells were incubated for 48 h. After incubation, the cells were harvested using trypsin-EDTA and centrifuged at 500g for 5 min. The cells were then washed with PBS and fixed with ice cold ethanol (70% in water). After ethanol fixation for 3 h at -20 °C, the cells were then centrifuged at 500g for 5 min. The ethanol was removed and the fixed cells were rinsed with PBS. The cell pellet was re-suspended in PBS (0.25 mL per 5×10^5 cells/well) and centrifuged after which the supernatant was discarded. The Muse™ cell cycle kit reagent (200 μ L) was added and the cells were re-suspended and incubated for 30 min in the dark at room temperature. The stained cells were

analyzed by flow cytometer (Muse™ cell analyzer). The DNA content was analyzed and the dot plot was recorded. A histogram of the DNA content index was generated and the cell populations were analyzed in each phase of the cycle. The cell cycle assay discriminated the cells using propidium iodide-based staining of the DNA content and measured the percentage of cells in each cell cycle phase (G0/G1, S and G2/M).

2.7 Primers design

The sequences of genes including *TNF- α* , *BCL-2*, *BAX*, *BAK*, and *GAPDH* were retrieved from GenBank Database (<https://www.ncbi.nlm.nih.gov/>). Oligonucleotides with 20-24 bp and theoretical melting temperatures ranging from 60 to 64 °C were designed accordingly, using Primer3 Software (<http://simgene.com/Primer3>). The list of primers is shown in Table 1.

2.8 Quantitative real-time PCR (qRT-PCR)

The human cancer cell lines (5×10^5 cells per well) in the 6-well plate were treated with plaunotol at concentrations of 50 μ M and 75 μ M and incubated further for 48 h at 37 °C in a 5% CO₂ humidified atmosphere. The cells were treated with 0.2% (v/v) DMSO and paclitaxel (1 μ M) as the negative and positive controls, respectively. After incubation, the cells were washed with PBS and treated with trypsin-EDTA. The suspension of cells was harvested by centrifugation at 500g for 5 min. The supernatant was removed and the cell pellet was rinsed with PBS and stored at -80 °C until use.

The total RNA was isolated from the cells using Total RNA Mini Kit (Geneaid, Taiwan) according to the manufacturer's protocol. The quality and quantity of total RNA of the treated cells were evaluated by UV spectrophotometer (Thermo Scientific, USA). The mRNA levels of genes were determined using qRT-PCR (ABI Prism® 7300) in the presence of RNA template and One Step SYBR® PrimeScript™ RT-PCR kit II (Perfect Real Time, Takara, Japan). The qRT-PCR was carried out in a final volume of 20 μ L reaction; with mixture containing 0.8 μ L of each primer (forward & reward) (Table 1), 10 μ L of 2x one step RT-PCR buffer IV, 0.4 μ L of ROX dye (50x), 2 μ L of RNA (20 ng) as a template and 0.8 μ L of PrimeScript one step enzyme mix II,

Table 1. Primers used in qRT-PCR experiment.

Name	Accession number* (location)	Sequence (5'→3')	Product size (bp)
F-TNF α	NM_000594.3	TGC TTG TTC CTC AGC CTC TTC TC	200
R-TNF α	(263-463)	AGG GTT TGC TAC AAC ATG GGC T	
F-BCL2	NM_000633.2	CCT GTG GAT GAC TGA GTA CCT GTG	129
R-BCL2	(1015-1144)	CAC AG AGA CAG CCA GGA GAA ATC A	
F-BAX	NM_001291428.1	GAG AGG TCT TTT TCC GAG TGG C	105
R-BAX	(335-440)	GCC TTG AGC ACC AGT TTG CTG	
F-BAK	NM_001188.3	GAG AGG TCT TTT TCC GAG TGG C	159
R-BAK	(1366-1525)	GCC TTG AGC ACC AGT TTG CTG	
F-GAPDH	NM_001256799.2	ACC CAC TCC TCC ACC TTT GAC	179
R-GAPDH	(1066-1245)	TCC TCT TGT GCT CTT GCT GG	

*Retrieved from <https://www.ncbi.nlm.nih.gov/>

and adjusted to the volume with RNase free water. At the optimal PCR condition, the amplification plot and melting curve were generated. The cycle number at threshold (set at 0.2) was recorded. The *GAPDH* gene was used as the endogenous gene. The expression level of the control treatment was used as a calibrator of each gene. The transcription profiles of the genes were expressed as relative quantitative (RQ), which was calculated using the comparative C_T method when $RQ = 2^{-\Delta\Delta C_T}$.

2.9 Statistical analysis

All data represented in this study are expressed as mean \pm SD. The experiments were performed in triplicate (n=3). Analysis of variance (ANOVA) followed by Dunnett's post-test was used to determine the significant differences between the groups, and P values ≤ 0.05 and ≤ 0.01 were considered significant at 95% and 99% confidence. All statistical analyses were conducted using IBM SPSS version 22 for Windows software.

3. Results

3.1 Effect of plaunotol on cell viability

The MTT assay was performed to determine the cell viability of plaunotol in human cancer cell lines. The growth of human cancer cell lines was inhibited when cells were treated with plaunotol in a concentration-dependent manner. The relationship between cell viability and concentration yielded the IC_{50} value. Table 2 summarizes the IC_{50} of plaunotol and paclitaxel against the cell lines. It can be concluded that plaunotol exhibits a moderate anti-proliferative activity.

3.2 Effect of plaunotol on cell division

To understand the action of plaunotol on anti-proliferative activity, the effect of plaunotol on cell division was performed by staining the DNA with propidium iodide. The cell cycle assay, using flow cytometer, indicated that the distribution of the cell population varied after treatment with plaunotol. The nuclear DNA intercalating stain propidium iodide discriminates cells at different stages of the cell cycle, based on differential DNA content. Figure 2 illustrates the histogram of cell population in different types of cell lines. The results indicated that plaunotol affects cell division of HT-29 at the G2/M phase, and MCF-7 at the S-phase. However, HeLa and KB had a slight effect at the G0/G1 phase with P values of 0.036 and 0.01, respectively (Figure 3).

3.3 Effect of plaunotol on apoptosis

In the treatment of the human cancer cell lines with plaunotol at 75 μ M and 150 μ M (equivalent to the IC_{50} and IC_{99} , respectively) for 48 h, the cells were prepared for double staining according to the manufacturer's protocol. After subjecting the mixture to the MuseTM analyzer, the population was gated. The results clearly showed that plaunotol altered the membrane from apoptosis (Figure 4A). This evidence appeared in each of the cancer cell types, although with different sensitivity. As shown in Figure 4B, plaunotol triggered apoptosis in the early phase. It can be noted that a high concentration of plaunotol had a cytotoxic effect on the HT-29.

3.4 Plaunotol caused apoptosis in both extrinsic and intrinsic pathways

After treating the human cancer cell lines with plaunotol (50 μ M and 75 μ M), the cells were harvested and the RNA extracted. The transcription profiles of apoptotic-associated genes, such as pro-apoptotic genes (*TNF- α* , *BAX*, and *BAK*) and anti-apoptotic genes (*BCL-2*), were determined when *GAPDH* was an endogenous gene. The relative expression was calculated as the RQ value according to the equation in the experiment.

In consideration of the expression profile of *TNF- α* , treatment of the cells with plaunotol increased the expression of *TNF- α* in HeLa and HT-29 (Figure 5A). In contrast, the *TNF- α* mRNA levels in MCF7 and KB cells were suppressed after treating the cells with plaunotol. The expressions of the anti-apoptotic gene *Bcl2* were decreased in each of the cell types. On the other hand, plaunotol did not affect *BAX* and *BAK* mRNAs in any of the cancer cell types. The ratio of expression level of *BCL-2* and *BAX* was estimated from the apoptotic-associate genes related to the apoptotic agent. The results in Figure 5B revealed that plaunotol caused apoptosis in HeLa, HT-29, and MCF7. In conclusion, plaunotol played an important role in altering cell division causing apoptosis by the suppression of apoptotic-associate genes via extrinsic and intrinsic pathways in HeLa, HT-29, and MCF7, but not in KB cells.

4. Discussion

Plaunotol or (*E,Z,E*)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecatetraen-1-ol was registered with the World Health Organization (WHO) under the name of CS-684 in 1983. It was manufactured in the form of a soft-gelatin capsule (combined with corn oil) under the tradename of

Table 2. Anti-proliferative activity of plaunotol against the human cancer cell lines (n=3).

Compound (conc.)	IC_{50} in different cell line*			
	HeLa	HT-29	MCF-7	KB
Plaunotol (μ M)	65.5 \pm 6.4	72.9 \pm 5.7	62.3 \pm 9.1	80.9 \pm 3.5
Paclitaxel (nM)	12.3 \pm 2.9	7.6 \pm 4.0	4.5 \pm 2.5	11.4 \pm 0.5

*The IC_{50} on the human gingival fibroblast was > 100 μ M.

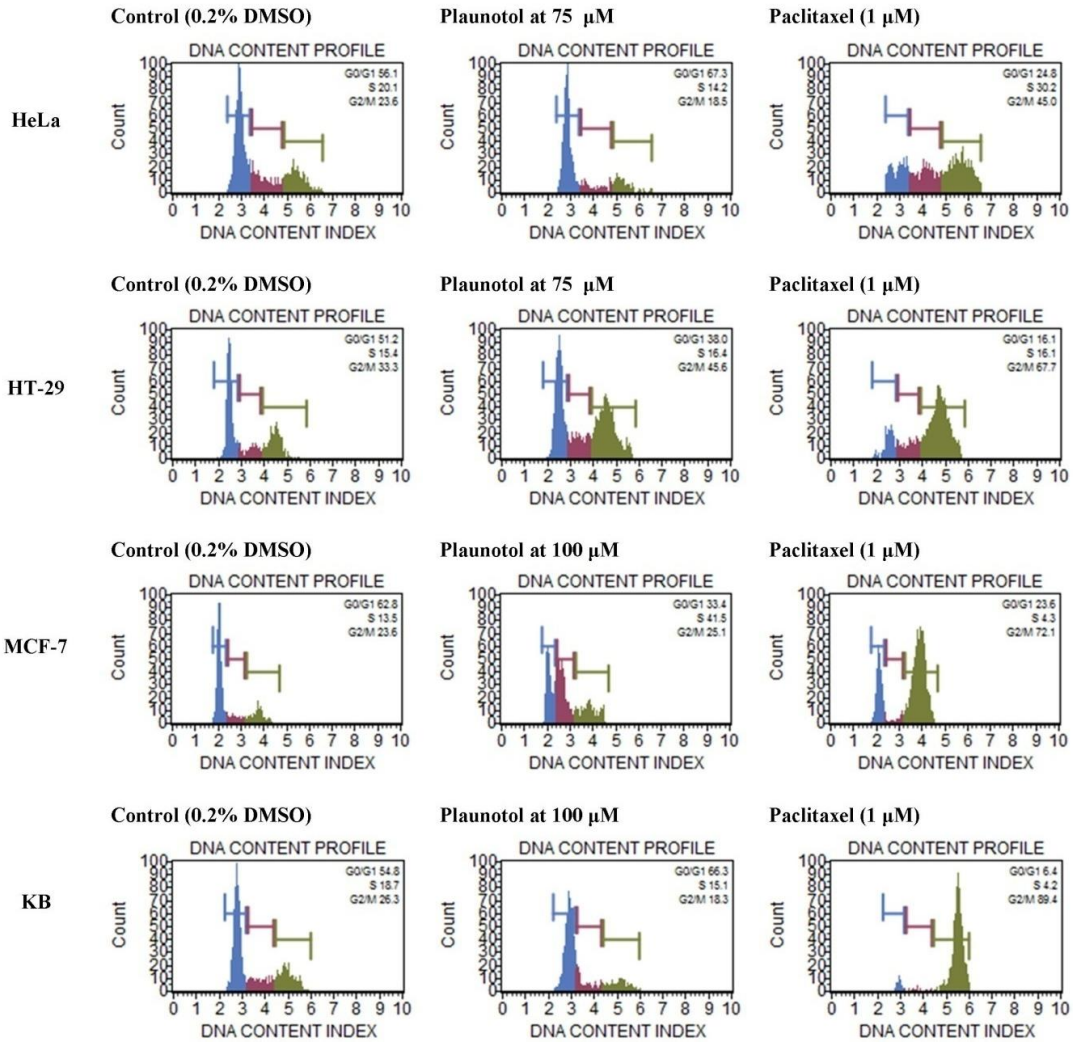


Figure 2. Histograms of DNA content profiles in the HeLa, HT-29, MCF7, and KB cells after treatment at 75 μM and 100 μM of plaunotol in comparison with control and paclitaxel treatments for 48 h. Cells were stained with propidium iodide and analyzed by flow cytometry.

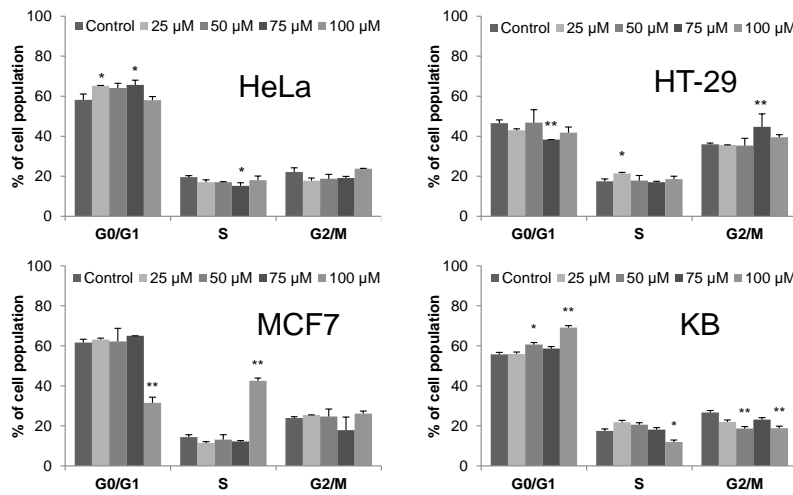


Figure 3. Percent of cell population of human cancer cell lines after treatment with different concentrations of plaunotol. Data are expressed as mean±SD of triplicate experiments and was analyzed by ANOVA followed by Dunnett's post-test where ** was at P<0.001 and * was at P<0.05 when compared to control.

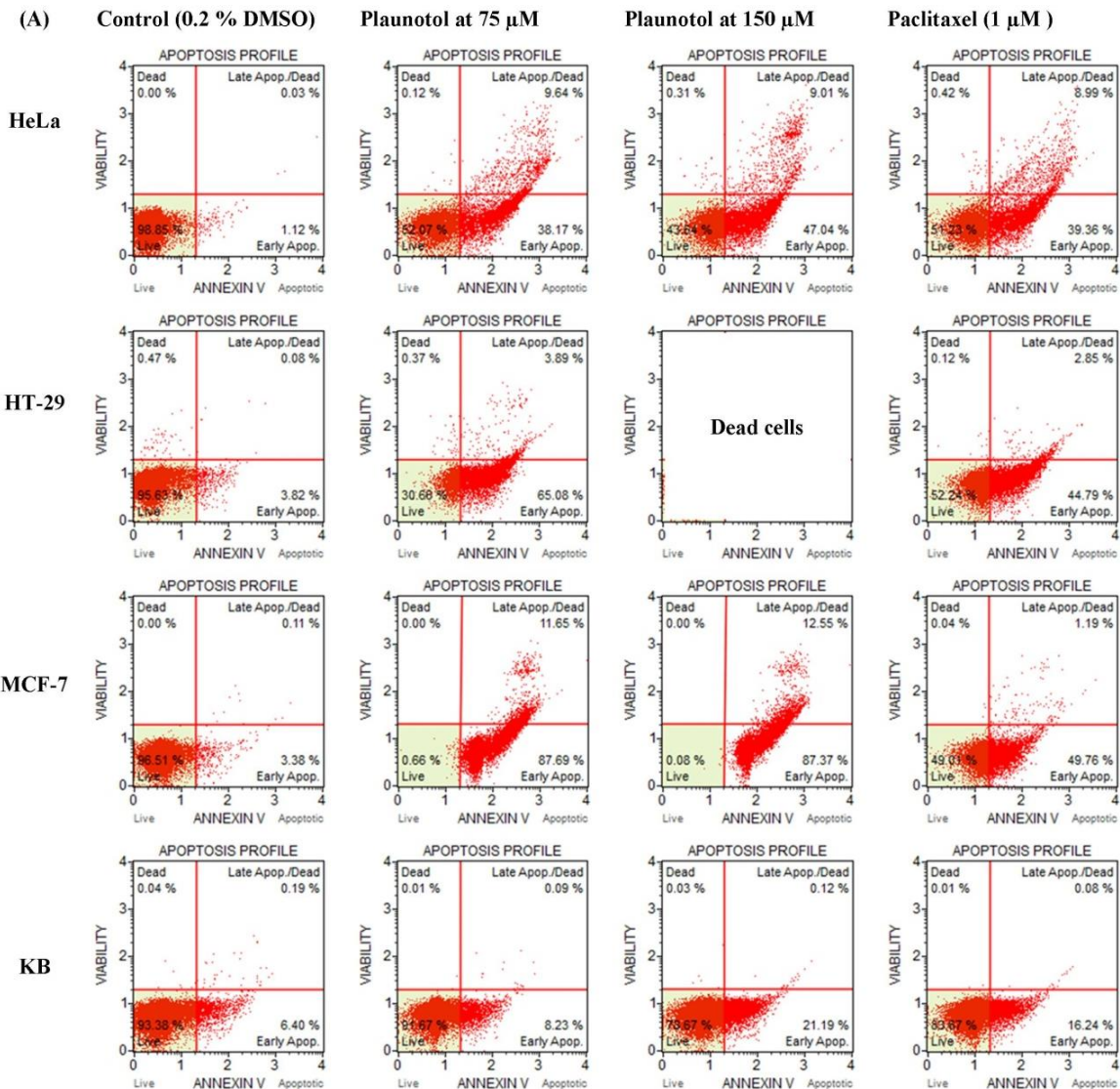


Figure 4A. Dot plots indicate amount of stained cells in each quadrant: live, early apoptosis, live cells, late apoptosis and dead cells, respectively in HeLa, HT-29, MCF7, and KB cells. Percentage of each quadrant indicates the population of cells obtained from gating.

Kelnac™ (Daiichi Sankyo Ltd., Tokyo, Japan). Since then, plaunotol has become an anti-peptic ulcer drug and is recommended by physicians to be combined with antibiotics and proton pump inhibitors for treatment of *Helicobacter pylori*-induced peptic ulcer (Takagi *et al.*, 2000).

In the last three decades, pharmacological activities of plaunotol have been investigated extensively with various publications. It possesses several pharmacological activities, such as anti-inflammation, gastro-protection, antibacterial activity, and anticancer properties. Plaunotol induces the production of prostaglandins in gastric mucosa that results in the release of endogenous secretin (Shiratori *et al.*, 1993) and suppresses the production of inflammatory mediators which are generated by leucocytes such as TNF- α and IL-8 (Murakmi *et al.*, 1999; Takagi *et al.*, 2000). For anticancer activity, in particular, only a few reports on the effect of plaunotol in cancer cells were found. Plaunotol was reported

to exert anti-cancer effects through its anti-angiogenic activity (Kawai *et al.*, 2005) and it has direct effects on gastric and colon cancer cells (Yamada *et al.*, 2007; Yoshikawa *et al.*, 2009). Therefore, in the interest of increasing the value of *C. stellatopilosus*, we proceeded to demonstrate the efficiency of plaunotol for anti-proliferative and apoptosis activities.

The anti-proliferative activity of plaunotol in the present study was reported against four types of human cancers: MCF-7; KB; HeLa; and HT-29. In parallel, human gingival fibroblast cells were used to represent normal cells. Evaluation of the cytotoxic activity using the MTT assay revealed that plaunotol exhibited dose-dependent anti-proliferative activity against all human cancer cell lines and expressed an IC₅₀ ranging from 60 μ M to 80 μ M. In addition, at tested concentrations (<100 μ M), plaunotol was not toxic to the HGF normal cell line. In DLD1-human colon adenocarcinoma, plaunotol and GGOH were shown to inhibit

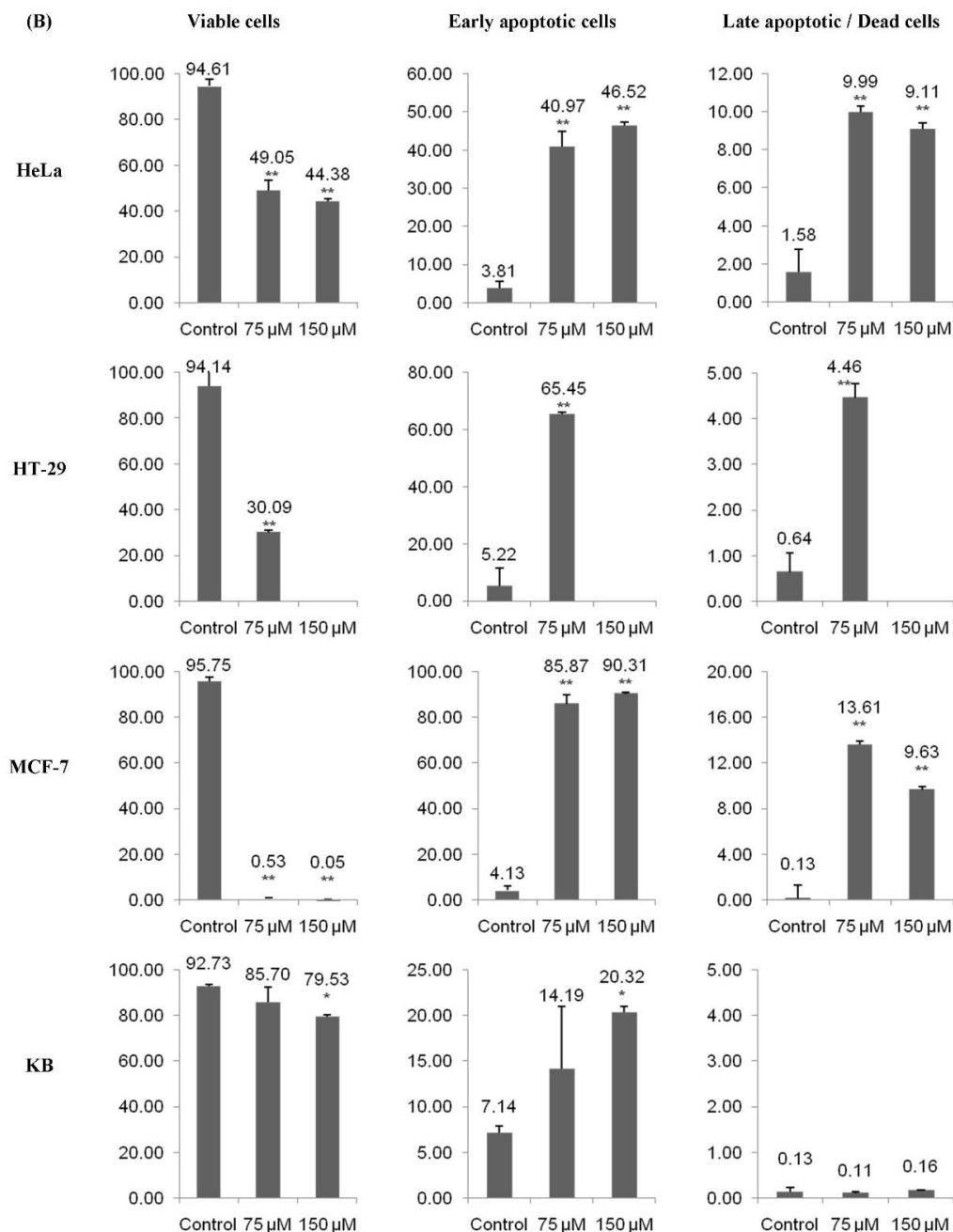


Figure 4B. Summary of the percent population of cells after treatment with plaunotol at 75 μ M and 150 μ M in different type of cancer cells in comparison with control (0.2% DMSO). The experiment was performed in triplicate. Data were analyzed by ANOVA followed by Dunnett's post-test (*, ** indicate P values <0.05 and <0.001, respectively).

growth of the cancer cell by inducing caspase-mediated apoptosis. These results showed that plaunotol not only inhibited growth of HT-29, a human colon cancer cell, but also MCF-7, KB, and HeLa cells.

Several anti-cancer drugs, such as paclitaxel, are known to inhibit cancer growth and block the cell cycle (Payne & Miles, 2008). The cell cycle is the control process in eukaryotic cells which evaluates the condition of the genetic formation during cell division. Its mechanism is regulated by three internal checkpoints including the G0/G1 phase, S-

phase, and G2/M phase. The G1 checkpoint is a major checkpoint caused by damage of DNA and the cell cannot pass to the next stage (S-phase). The S checkpoint checks the replication of DNA prior to undergoing the mitosis stage. The last stage, M checkpoint, evaluates whether all sister chromatids are correctly attached to spindle microtubules before the cell enters the irreversible anaphase. From the results of the study, the human cancer cell lines had different responses to plaunotol at 75 and 100 μ M. Plaunotol exhibited anti-proliferative activity at the resting stage (G0/G1) of HeLa

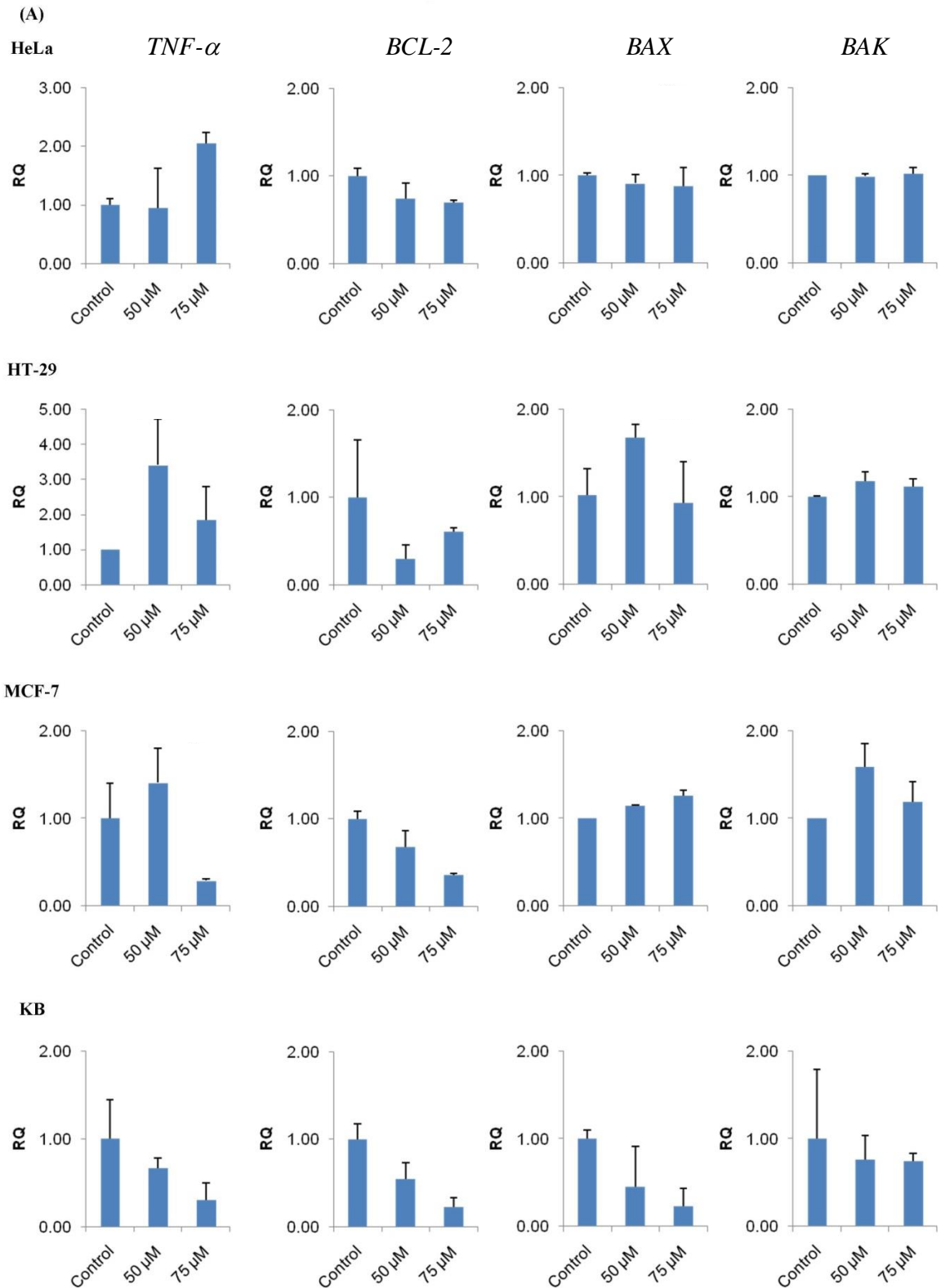


Figure 5. The relative expression levels of apoptotic-associate genes in the human cancer cell lines (HeLa, HT-29, MCF-7, and KB cells) after treatment with plaunotol at 50 μ M and 75 μ M for 48 h. (A) the RQ values of *TNF- α* , *BCL-2*, *BAX* and *BAK*. (B) the RQ ratios of RQ_{BCL-2} and RQ_{BAX} .

(B)

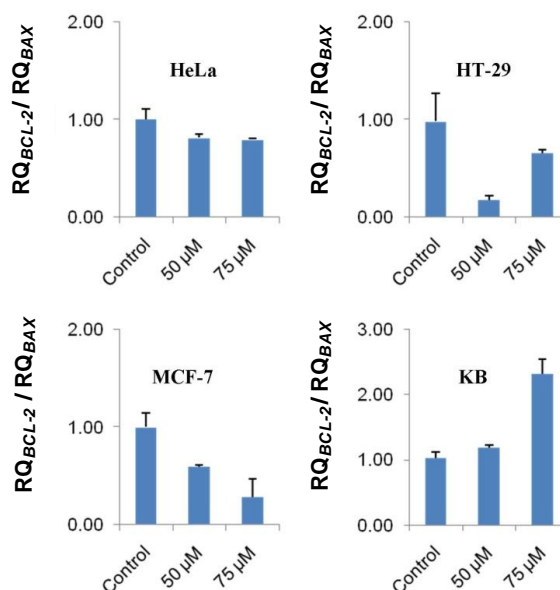


Figure 5. Continued.

and KB. On the other hand, it caused an inhibition during DNA synthesis (S) of MCF-7 and during cell division (G2/M) of HT-29. It can be concluded that plaunotol has anti-proliferative activity that affects cell division at different stages depending upon the type of cancer cell.

The inhibitory effect of plaunotol against HeLa, HT-29, MCF-7, and KB-cells was confirmed by staining the cells with Annexin V. The results indicated that HeLa, HT-29, and MCF-7 were sensitive to plaunotol at 75 and 150 μ M and induced apoptosis at the early stage of apoptosis. In contrast, plaunotol had less effect on KB-cells, which is a derivative of HeLa cells. A different mechanism of plaunotol on anti-proliferative activity in the KB-cells can be postulated.

Plaunotol has been reported to induce apoptosis of gastric cancer cells (Yamada *et al.*, 2007). Moreover, it activated caspases and induced apoptosis in colon cancer (Yoshikawa *et al.*, 2009). The present study clearly showed that plaunotol acted as an apoptotic agent in breast cancer, as well as cervix and colon cancers. It blocked the cell cycle during cell division, altered the cell membrane leading to cell death and inhibited *BCL-2* expression which resulted in apoptosis of the cancer cells. In summary, plaunotol induces apoptosis through the death receptor and mitochondrial dependent pathway. In summary, the present study provided supportive data on the anti-proliferative activity and apoptotic mechanism of plaunotol in human cancer cells. Thus, plaunotol may have therapeutic potential in chemotherapy for the treatment of human cancer.

References

- Chaotham, C., Chivapat, S., Chaikitwattana, A., & De-Eknamkul, W. (2013). Acute and chronic oral toxicity of a partially purified plaunotol extract from *Croton stellatopilosus* Ohba. *BioMed Research International*, 1-12. doi:10.1155/2013/303162
- Cooper, G. M. (2000). *The cell: A molecular approach*. Washington, D.C: ASM Press.
- Fuchs, Y. & Steller, H. (2011). Programed cell death in animal development and disease. *Cell*, 147(4), 742-758. doi:10.106/j.cell.2011.10.033
- Grynberg, N. F., Echevarria, A., Lima, J. E., Pamplona, S. S. R., Pinto, A. C., & Maciel, M. A. M. (1999). Antitumour activity of two 19-nor-clerodane diterpenes, *trans*-dehydrocrotonin and *trans*-crotonin, from *Croton cajucara*. *Planta Medica*, 65(8), 687-689. doi:10.1055/s-1999-14042
- Jo, A. R., Jeong, H. S., Kim, M. K., Yun, H. Y., Baek, K. J., Kwon, N. S., & Kim, D. S. (2016). Geranylgeranylacetone induces apoptosis via intrinsic pathway in human melanoma cells. *Biomedicine and Pharmacotherapy*, 82, 15-19. doi:10.1016/j.biopha.2016.04.051
- Joo, J. H., Liao, G., Collins, J. B., Grissom, S. F., & Jetten, A. M. (2007). Farnesol-induced apoptosis in human lung carcinoma cells is coupled to the endoplasmic reticulum stress response. *Cancer Research*, 67(16), 7929-7936. doi:10.1158/0008-5472.CAN-07-0931
- Kawai, K., Tsuno, N. H., Kitayama, J., Okaji, Y., Yazawa, K., Asakage, M., . . . Nagawa, H. (2005). Anti-angiogenic properties of plaunotol. *Anticancer Drugs*, 16(4), 401-407.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *Journal of Immunological Methods*, 65(1-2), 55-63. doi:10.1016/0022-1759(83)90303-4
- Murakami, K., Okajima, K., Harada, N., Isobe, H., Liu, W., Johno, M., & Okabe, H. (1999). Plaunotol prevents indomethacin-induced gastric mucosal injury in rats by inhibiting neutrophil activation. *Alimentary Pharmacology and Therapeutics*, 13(4), 521-530.

- Nakamura, N., Shidoji, Y., Yamada, Y., Hatakeyama, H., Moriwaki, H., & Muto, Y. (1995). Induction of apoptosis by acyclic retinoid in the human hepatoma-derived cell line, huh-7. *Biochemical and Biophysical Research Communications*, 207(1), 382-388. doi:10.1006/bbrc.1995.1199
- Ogiso, A., Kitazawa, E., Kurabayashi, M., Sato, A., Takahashi, M., Noguchi, H., Kuwano, H., Kobayashi, S., & Mishima, H. (1978). Isolation and structure of anti-peptic ulcer diterpene from Thai medicinal plant. *Chemical and Pharmaceutical Bulletin*, 26(10), 3117-3123. doi:10.1248/cpb.26.3117
- Ogiso, A., Kitazawa, E., Kobayashi, S., Komai, T., Matsunuma, N., & Kataumi, S. (1985). Plaunotol (CS-684), a new anti-ulcer agent. *Sankyo Kenyusho Nempo*, 37,1-39.
- Ohizumi, H., Masuda, Y., Nakajo, S., Sakai, I., Ohsawa, S., & Nakaya, K. (1995). Geranylgeraniol is a potent inducer of apoptosis in tumor cells. *Journal of Biochemistry*, 117(1), 11-13.
- Park, J. S., Kwon, J. K., Kim, H. R., Kim, H. J., Kim, B. S., & Jung, J. Y. (2014). Farnesol induces apoptosis of DU145 prostate cancer cells through the PI3K/Akt and MAPK pathways. *International Journal of Molecular Medicine*, 33(5), 1169-1176. doi:10.3892/ijmm.2014.1679
- Payne, S., & Miles, D. (2008). Mechanisms of anticancer drugs. In M. Gleeson (Ed.), *Scott-Brown's otorhinolaryngology, head and neck surgery* (pp. 34-46). Florida, FL: CR press.
- Prayong, P., Weerapreeyakul, N., & Barusrux, S. (2007). Apoptosis: Pathway and assay. *Journal of Health Research*, 21(3), 227-238.
- Roengsumran, S., Singtothong, P., Pudhom, K., Ngamrochana vanich, N., Petsom, A. & Chaichantipyuth, C. (1999). Neocrotovembranal from *Croton oblongifolius*. *Journal of Natural Products*, 62(8), 1163-1164. doi:10.1021/np9900268
- Santos, H. S., Barros, F. W., Albuquerque, M. R., Bandeira, P. N., Pessoa, C., Braz-Filho, R., . . . Lemos, T. L. (2009). Cytotoxic diterpenoids from *Croton argyrophyllodes*. *Journal of Natural Products*, 72(10), 1884-1887. doi:10.1021/np900250k
- Shidoji, Y., Nakamura, N., Moriwaki, H., & Muto, Y. (1997). Rapid loss in the mitochondrial membrane potential during geranylgeranoic acid-induced apoptosis. *Biochemical and Biophysical Research Communications*, 230(1), 58-63. doi:10.1006/bbrc.1996.5883.
- Shidoji, Y., Okamoto, K., Muto, Y., Komura, S., Ohishi, N., & Yagi, K. (2006). Prevention of geranylgeranoic acid-induced apoptosis by phospholipid hydroperoxide glutathione peroxidase gene. *Journal of Cellular Biochemistry*, 97(1), 178-187. doi:10.1002/jcb.20627.
- Shiratori, K., Watanabe, S., Takeuchi, T. (1993). Role of endogenous prostaglandins in secretin- and plaunotol-induced inhibition of gastric acid secretion in the rat. *The American Journal of Gastroenterology*, 88(1), 84-89.
- Takagi, A., Koga, Y., Aiba, Y., Kabir, A. M., Watanabe, S., Ohta-Tada, U., . . . Miwa, T. (2000). Plaunotol suppresses interleukin-8 secretion induced by *Helicobacter pylori*: therapeutic effect of plaunotol on *H. pylori* infection. *Journal of Gastroenterology and Hepatology*, 15(4), 374-380. doi:10.1046/j.1440-1746.2000.02168.x.
- Ushiyama, S., Matsuda, K., Asai, F., & Yamazaki, M. (1987). Stimulation of prostaglandin production by (2E,6Z,10E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecatetraen-1-ol (plaunotol), a new antiulcer drug, *in vitro* and *in vivo*. *Biochemical Pharmacology*, 36(3), 369-375.
- Vermes, I., Haanen, C., Steffens-Nakken, H., & Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunological Methods*, 184(1), 39-51. doi:10.1016/0022-1759(95)00072-I.
- Yamada, J., Kawai, K., Tsuno, N. H., Kitayama, J., Tsuchiya, T., Yoneyama, S., . . . Nagawa, H. (2007). Plaunotol induces apoptosis of gastric cancer cells. *Planta Medica*, 73(10), 1068-1073. doi:10.1055/s-2007-981578.
- Yoshikawa, N., Yamada, J., Tsuno, N. H., Okaji, Y., Kawai, K., Tsuchiya, T., . . . Takahashi, K. (2009). Plaunotol and geranylgeraniol induce caspase-mediated apoptosis in colon cancer. *The Journal of Surgical Research*, 153(2), 246-253. doi:10.1016/j.jss.2008.04.021.
- Yoshikawa, N., Tsuno, N. H., Okaji, Y., Kawai, K., Shuno, Y., Nagawa, H., . . . Takahashi, K. (2010). Isoprenoid geranylgeranylacetone inhibits human colon cancer cells through induction of apoptosis and cell cycle arrest. *Anticancer Drugs*, 21(9), 850-860. doi: 10.1097/CAD.0b013e32833e53cf.