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

*Corresponding author.

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

Plaunotol, an acyclic diterpene alcohol, was evaluated for an anti-proliferative activity in human cancer cell lines including HeLa, HT-29, MCF-7 and KB cells. After treatment cells with plaunotol, cell viability was determined using MTT assay. The results showed that plaunotol inhibited the growth of human cancer cell lines with IC$_{50}$ of 65.47 ± 6.39 µM (HeLa), 72.92 ± 5.73 µM (HT-29), 80.90 ± 3.48 µM (KB) and 62.25 ± 9.15 µM (MCF-7), respectively. For apoptotic detection, treated cells 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 $\text{TNF-}\alpha$, $\text{BCL-2}$, $\text{BAX}$ and $\text{BAK}$ genes was determined using qRT-PCR technique and the expression level was calculated as the relative quantitation (RQ). The ratio between RQ of $\text{BCL-2}$ and $\text{BAX}$ 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 (Fig. 1) is an acyclic diterpene alcohol derived from geranylgeranyl diphosphate (GGPP). It has been isolated since 1978 and was only found in *Croton stellatopilosus* Ohba or plaunoi [Thai name] (Ogiso *et al.*, 1978). Plaunotol was claimed to have anti-peptic ulcer ability and it has been 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 test in animal model (Chaotham, Chivapat, Chaikitwattana, & De-Eknamkul, 2013).

Plaunotol has gastro-protective effect that mediates several mechanisms such as releasing prostaglandins (PGE$_2$ and PGI$_2$), secretin and inhibiting neutrophil activation (Ushiyama *et al.*, 1987; Shiratori, Watanabe, & Takeuchi, 1993; Murakami *et al.*, 1999). Considering *Croton* species, some diterpenoids have been reported for 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), *ent*-15-oxo-kaur-16-en-18-oic acid from the bark of *C. argyrophylloides* (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, Nakamura, Moriwaki, & Muto, 1997;
Shidoji et al., 2006) and geranylgeranylacetone (Yoshikawa et al., 2010; Jo et al., 2016), have been reported to inhibit cancer cell growth via apoptosis induction.

Plaunotol has been identified as a cytoprotective anti-peptic ulcer agent. Up to date, plaunotol has been 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 cytotoxic effect against 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 the network of regulation at the molecular level of cell growth and cell division. Apoptosis is a biological process in multi-cellular organism that plays an important role in animal development and homeostasis (Fuchs & Steller, 2011). During apoptosis, the cell initially defined by its biochemical characteristic changes, including cell shrinkage, blebbing of the plasma membrane, nuclear morphology (which includes the chromatin condensation and fragmentation, and releasing the apoptotic mediators) (Prayong, Weerapreeyakul, & Barusrux, 2007). 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, cell cycle process is a failure and results in an uncontrolled cell proliferation. For this reason, a better understanding of cancer biology and cancer genetics is necessary for cancer research. Among these researches, many reports were focused on the anti-proliferative effect of cancer cells, its mechanisms and the realization that cell cycle progression, apoptosis and regulatory genes involve apoptosis and cell cycle.
Based on the above mentioned 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 the investigation of molecular mechanism of plaunotol on anti-proliferative effect, induction of apoptosis and accumulation of cells in cell cycle phase. In addition, the transcription analysis of associated-apoptotic mediators on four cancer cell lines including 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 as described in Premprasert *et al.* (2013). The structure was confirmed by spectroscopic methods [\(^1\)H-, \(^{13}\)C-NMR and MS].

Plaunotol: a pale yellow oil; IR \(\nu_{\text{max}}\) cm\(^{-1}\) 3300 (O−H), 1665 (C=O), 1440 (C−H), 1380, 1000; \(^1\)H NMR (CDCl\(_3\), 500 MHz) \(\delta\): 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); \(^{13}\)C NMR (CDCl\(_3\), 125 MHz) \(\delta\): 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 was from Gibco® BRL, CA, USA. The antibiotics [Penicillin G (100 U/mL) plus streptomycin (100 µg/mL)] was supplied by Invitrogen®, CA, 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 form 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 mono-layered cells were sub-cultured weekly with 0.25% trypsin-EDTA when 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 96-well plate. These cells were attached to a plate after 24 h incubation at 37°C in an atmosphere of 5% CO_2. Aliquots of medium containing different concentrations of plaunotol (3, 10, 30 and 100 µM) were added and the cell cultures 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. The 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, Backman Coulter Inc, Austria) using a test wavelength of 570 nm. The correlation between concentration and % inhibition was interpreted for IC_{50} value in micromolar unit.

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). Human cancer cell lines: HeLa, HT-29, KB and MCF-7 were separately seeded briefly at 5×10^5 cells per wells and treated with 75 µM and 150 µM of plaunotol for 48 h. Paclitaxel at concentration of 1 µM was used as positive control. After treatment, cells were washed twice with PBS and harvested with 0.25% trypsin-EDTA, followed by centrifugation at 500×g for 5 min. Then, cell pellet was re-
suspended in 100 µl of DMEM media containing 1% FBS followed by the addition of 100 µl of Muse™ Annexin V et Dead cell reagent. The solution was incubated for 20 min at room temperature in dark condition. Finally, the stained cells were analyzed by flow cytometer using Muse™ cell analyzer. The data was analyzed using Muse™ 1.4 software and the data was shown as four-quadrant dot plot. The statistic on four cell populations was obtained and the populations in each quadrant predicted the apoptosis in live, early apoptotic, late apoptotic and dead, respectively.

2.6 Cell cycle analysis

HeLa, HT-29, KB and MCF-7 cancer cells were seeded into 6-well plate at 5×10^5 cells/well, then cells were treated with varying doses of plaunotol (25 µM to 100 µM) and with DMSO (0.2%) as a control group. Treated cells were incubated for 48 h. After incubation, the cells were harvested using trypsin-EDTA and centrifuged at 500×g for 5 min. Then, the cells were 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 500×g for 5 min. Ethanol was removed and the fixed cell was rinsed with PBS. The cell pellet was re-suspended in PBS (0.25 mL per 5×10^5 cells/well), centrifuged after which the supernatant was discarded. The Muse™ cell cycle kit reagent (200 µL) was added; 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. The histogram of the DNA content index was generated and analyzed the cell populations in each phase of the cycle; The cell cycle assay exploited propiodium iodide-based staining of 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 \textit{TNF-\(\alpha\), BCL-2, BAX, BAK} and \textit{GAPDH} were retrieved from GenBank Database (https://www.ncbi.nlm.nih.gov/).

Oligonucleotides with 20-24 bp and theoretical melting temperature ranging from 60 - 64 °C were designed accordingly, using Primer3 Software (http://simgene.com/Primer3). List of primers are shown in Table 1.

2.8 Quantitative real-time PCR (qRT-PCR)

The human cancer cell lines (5×10\(^5\) cells per well) in 6-well plate were treated with plaunotol at concentrations of 50 \(\mu\)M and 75 \(\mu\)M and incubated further for 48 h at 37°C in a 5% CO\(_2\) humidified atmosphere. The cells were treated with 0.2% (v/v) DMSO and paclitaxel (1 \(\mu\)M) as negative control and positive control, respectively.

After incubation, the cells were washed with PBS and treated with trypsin-EDTA. The suspension of cells was harvested by centrifugation at 500×g for 5 min. 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 each treated cells were evaluated by UV spectrophotometer (Thermo Scientific, USA). The mRNA levels of genes were determined using qRT-PCR (ABI Prism\textsuperscript{\textregistered} 7300) in the presence of RNA template and One Step SYBR\textsuperscript{\textregistered} PrimeScript\textsuperscript{TM} RT-PCR kit II (Perfect Real Time, Takara, Japan). The qRT-PCR was carried out in a final volume of 20 \(\mu\)L reaction; with mixture containing 0.8 \(\mu\)L of each primer (forward & reward) (Table 1), 10 \(\mu\)L of 2x one step RT-PCR buffer IV, 0.4 \(\mu\)L of ROX dye (50x), 2 \(\mu\)L of RNA (20 ng) as a template and 0.8 \(\mu\)L of PrimeScript one step enzyme mix II, and
adjusted to the volume with RNase free water. At optimal PCR condition, the amplification plot and melting curve were generated. Cycle number at threshold (set at 0.2) was recorded. The \textit{GAPDH} gene was used as endogenous gene. Expression level of control treatment was used as a calibrator of each gene. The transcription profile of gene was 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 the data represented in this study are expressed as mean ± S.D. 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 groups, and \(P\) value ≤ 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 the 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 afforded 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 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. Fig. 2 illustrates the histogram of cell population in different types of cell lines. The results indicate that plaunotol affects cell division of HT-29 at G2/M phase, and MCF-7 at S-phase. However, two types of cell lines like HeLa and KB have slightly increased effect at G0/G1 phase with P-values of 0.036 and 0.01, respectively, as shown in Fig. 3.

3.3 Effect of plaunotol on apoptosis

In treatment of the human cancer cell lines with plaunotol at 75 µM and 150 µM (equivalent to the IC50 and IC99, respectively) for 48 h, cells were prepared for double staining according to the manufacturer’s protocol. After being subjected the mixture to the Muse™ analyzer, the population was gated. The results clearly showed that plaunotol altered the membrane from apoptosis (Fig. 4A). This evidence appeared in every cancer cell types, although with different sensitivity. As shown in Fig. 4B, plaunotol triggered apoptosis in the early phase. It can be noted that high concentration of plaunotol has cytotoxic effect in HT-29.

3.4 Plaunotol caused apoptosis in both extrinsic and intrinsic pathways
After treating the human cancer cell lines with plaunotol (50 µM and 75 µM), 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 mentioned equation in the experiment.

In consideration of the expression profile of TNF-α, as shown in Fig. 5A, treatment of cells with plaunotol increased the expression of TNF-α in HeLa and HT-29. In contrast, TNF-α mRNA levels in MCF7 and KB cells were suppressed after treating cells with plaunotol. For anti-apoptotic gene Bcl2, the expressions were decreased in every cell type. On the other hand, plaunotol did not affect BAX and BAK mRNAs in all 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 result in Fig. 5B revealed that plaunotol caused apoptosis in HeLa, HT-29 and MCF7. In conclusion, plaunotol play an important role in altering the cell division, causing apoptosis by the suppression of apoptotic-associate genes via extrinsic and intrinsic pathway 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 has been registered with the World Health Organization (WHO), under the name of CS-684 since 1983. It was manufactured in the form of soft-gelatin capsule (combined with corn oil) under the tradename of Kelnaç™ (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 prostaglandins production in gastric mucosa, releasing 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 few reports on the effect of plaunotol in the cancer cells were found. Plaunotol was reported to exert anti-cancer effect through its anti-angiogenic activity (Kawai et al., 2005) and direct effect on gastric and colon cancer cells (Yamada et al., 2007; Yoshikawa et al., 2009). Therefore, in the highlight of increasing value of C. stellatopilosus, we proceeded to demonstrate the efficiency of plaunotol for anti-proliferative and apoptosis activities.

The present study, anti-proliferative activity of plaunotol was reported against four types of human cancers including human breast carcinoma cell line (MCF-7), human cervical carcinoma cell line (KB), human cervix adenocarcinoma (HeLa), and human colorectal adenocarcinoma (HT-29). In parallel, the human gingival fibroblast was used represent a normal cell. Evaluation of cytotoxic activity with MTT assay revealed that plaunotol exhibited dose-dependent anti-proliferative activity against all human cancer cell lines and expressed an IC_{50} ranging from 60 µM to 80 µM. In addition, at tested concentrations (< 100 µM), plaunotol was not toxic to normal cell
line (HGF). In DLD1-human colon adenocarcinoma, plaunotol and geranylgeraniol (GGOH) were shown to inhibit 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.

In consideration of cell cycle arrest, several anti-cancer drugs are known for inhibiting cancer growth and blocking cell cycle (Payne & Miles, 2008), like paclitaxel, for instance. The cell cycle is the control process in eukaryotic cell, which evaluate the condition of the genetic formation during cell division. Its mechanism is regulated by three internal checkpoints including G0/G1 phase, S phase, and G2/M phase. G1 checkpoint is a major checkpoint caused by damage of DNA and the cell cannot pass to the next stage (S phase). 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, the human cancer cell lines have different response to plaunotol at 75 and 100 µM. Plaunotol exhibited anti-proliferative activity at the resting stage (G0/G1) of HeLa 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 an anti-proliferative activity, affecting the 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 result indicated that HeLa, HT-29 and MCF-7 were sensitive to plaunotol at 75 and 150 µM and induced at 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 membrane of the cells causing cell death and inhibited the $\textit{BCL-2}$ expression, resulting in apoptosis of the cancer cells. In summary, plaunotol induces apoptosis through death receptor and mitochondrial dependent pathway. Altogether, the present study provided supportive data on the anti-proliferative activity and apoptotic mechanism of plaunotol in human cancer cells. Thus, plaunotol may have a therapeutic potential or exert on chemotherapy for the treatment of human cancer.

**Acknowledgements**

This work was funded from the Annual Government Statement of Expenditure, Prince of Songkla University (PSU); under Grant number PHA580237S; the PSU Graduate School; and the Thailand Research Fund through the Golden Jubilee PHD program under Grant number PHD/071/2553)

**References**


Yoshikawa, N., Yamada, J., Tsuno, N.H., Okaji, Y., Kawai, K., Tsuchiya, T.,
Yoneyama, S., Tanaka, J., Shuno, Y., Nishikawa, T., Nagawa, H., Oshima, N.,
& Takahashi, K. (2009). Plaunotol and geranylgeraniol induce caspase-

Yoshikawa, N., Tsuno, N.H., Okaji, Y., Kawai, K., Shuno, Y., Nagawa, H., Oshima, N.,
& 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.
Figure caption

Figure 1. Chemical structure of plaunotol.

Figure 2. Histograms of DNA content profiles in the HeLa, HT-29, MCF7 and KB cells after treatment at 75 µM and 100 µM plaunotol in comparison with control and paclitaxel treatments for 48 h. Cells were stained with propiodium 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 were expressed as mean ± S.D 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.

Figure 4B. Summary of the percent population of cells after treatment with plaunotol at 75 µM and 150 µM 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 value < 0.05 and < 0.001, respectively).

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 treated with plaunotol at 50 µM and 75 µM 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}. 
Figure 1
Figure 2
Figure 3
Figure 4A
<table>
<thead>
<tr>
<th></th>
<th>Viable cells</th>
<th>Early apoptotic cells</th>
<th>Late apoptotic / Dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>94.61 ± 49.05</td>
<td>40.97 ± 46.52 **</td>
<td>9.99 ± 9.11 **</td>
</tr>
<tr>
<td></td>
<td>44.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-29</td>
<td>94.14 ± 30.09</td>
<td>66.45 **</td>
<td>4.46 **</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>95.75 ± 0.53</td>
<td>85.87 ± 90.31 **</td>
<td>13.61 ± 9.63 **</td>
</tr>
<tr>
<td></td>
<td>0.05 ± 4.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KB</td>
<td>92.73 ± 85.70</td>
<td>14.19 ± 20.32</td>
<td>0.13 ± 0.16</td>
</tr>
</tbody>
</table>

Figure 4B
Figure 5
Figure 5
Table 1. Primers used in qRT-PCR experiment.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession number*</th>
<th>Sequence (5′→3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLTNFα</td>
<td>NM_000594.3</td>
<td>TGC TTG TTC CTC AGC CTC TTC TC</td>
<td>200</td>
</tr>
<tr>
<td>R-TNFα</td>
<td>(263-463)</td>
<td>AGG GTT TGC TAC AAC ATG GGC T</td>
<td></td>
</tr>
<tr>
<td>F-BCL2</td>
<td>NM_000633.2</td>
<td>CCT GTG GAT GAC TGA GTA CCT GTG</td>
<td>129</td>
</tr>
<tr>
<td>R-BCL2</td>
<td>(1015-1144)</td>
<td>CAC AGA CAG CCA GGA GAA ATC A</td>
<td></td>
</tr>
<tr>
<td>F-BAX</td>
<td>NM_001291428.1</td>
<td>GAG AGG TCT TTT TCC GAG TGG C</td>
<td>105</td>
</tr>
<tr>
<td>R-BAX</td>
<td>(335-440)</td>
<td>GCC TTG AGC ACC AGT TTG CTG</td>
<td></td>
</tr>
<tr>
<td>F-BAK</td>
<td>NM_001188.3</td>
<td>GAG AGG TCT TTT TCC GAG TGG C</td>
<td>159</td>
</tr>
<tr>
<td>R-BAK</td>
<td>(1366-1525)</td>
<td>GCC TTG AGC ACC AGT TTG CTG</td>
<td></td>
</tr>
<tr>
<td>F-GAPDH</td>
<td>NM_001256799.2</td>
<td>ACC CAC TCC TCC ACC TTT GAC</td>
<td>179</td>
</tr>
<tr>
<td>R-GAPDH</td>
<td>(1066-1245)</td>
<td>TCC TCT TGT GCT CTT GCT GG</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ in different cell line*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(conc.)</td>
<td>HeLa</td>
</tr>
<tr>
<td>Plaunotol (µM)</td>
<td>65.5±6.4</td>
</tr>
<tr>
<td>Paclitaxel (nM)</td>
<td>12.3±2.9</td>
</tr>
</tbody>
</table>

*The IC$_{50}$ on the human gingival fibroblast was > 100 µM.