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Effects of Triterpenoids from *Luvunga scandens* on Cytotoxic, Cell Cycle Arrest and Gene Expressions in MCF-7 Cells

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Abstract – Plant-derived triterpenoids commonly possesses biological properties such as anti-inflammatory, anti-microbial, anti-viral and anti-cancer. *Luvunga scandens* is one of the plant that produced triterpenoids. The aims of the study was to analyze cell cycle profile and to determine the expression of p53 unregulated modulator of apoptosis (*PUMA*), *caspase-8* and *caspase-9* genes at mRNA level in MCF-7 cell line treated with two triterpenoids, flindissol (**1**) and 3-oxotirucalla-7,24-dien-21-oic-acid (**2**) isolated from *L. scandens*. The compounds were tested for cell cycle analysis using flow cytometer and mRNA expression level using quantitative RT-PCR. The number of MCF-7 cells population which distributed in Sub G_1 phase after treated with compound **1** and **2** were 7.7 and 9.3% respectively. The evaluation of the expression of genes showed that both compounds exhibited high level of expression of *PUMA, caspase-8* and *caspase-9* as normalized to *β-actin* via activation of those genes. In summary, the isolated compounds of *L. scandens* plant showed promising anticancer properties in MCF-7 cell lines.
Keywords – *Luvunga scandens*, Triterpenoids, MCF-7, Gene expressions, Flow cytometry

Introduction

Breast cancer is one of the most frequently diagnosed cancers and became a second leading cause of death among women after lung cancer.[1,2] Breast cancer is closely related with the lack or complete loss of critical component of *caspase-3*. *Caspase-3* plays a key role in the death receptor pathway, which initiated by *caspase-8* and the mitochondrial pathway which involving *caspase-9*.[3] Therefore, the execution phase of apoptosis involves the activation of a series of caspases which are *caspase-9* as the upstream caspase for the intrinsic pathway while *caspase-8* in extrinsic pathway and *caspase-3* in both intrinsic and extrinsic pathways. However, normal expression level of *caspase-8* and *caspase-9* were shown in MCF-7 cells. *PUMA* also plays an important role in apoptosis process. *PUMA* known as p53 unregulated modulator of apoptosis, is a potent pro-apoptotic molecule which is initially identified as a gene activated by p53 in cells undergoing p53 induced apoptosis. All genes used in this study are the pro-apoptotic which induced apoptosis. *PUMA*, *caspase-8* and *caspase-9* genes were used in this study except for *caspase-3* because the MCF-7 cell line does not express *caspase-3* due to deletion of *cas-3* gene.[4]

The plant-derived triterpenoids commonly used due to its ability to exhibit cytotoxicity against a variety of tumor cells. Our previous publication reported isolation of flindissol (compound **1**) (Fig. 1(a)) and 3-oxotirucalla-7,24-dien-21-oic acid (compound **2**) (Fig. 1(b)) from *L. scandens* exhibited cytotoxic activity assayed by MTT technique. It was found that the inhibitory activities (IC_{50}) of compound **1** and **2** were 13.8 μM and 27.5 μM, respectively.[5] In this paper, the study has been extended with the flow cytometric analysis of apoptotic cells and quantitative RT-PCR to determine cell cycle regulation after treatment with compound **1** and **2**.

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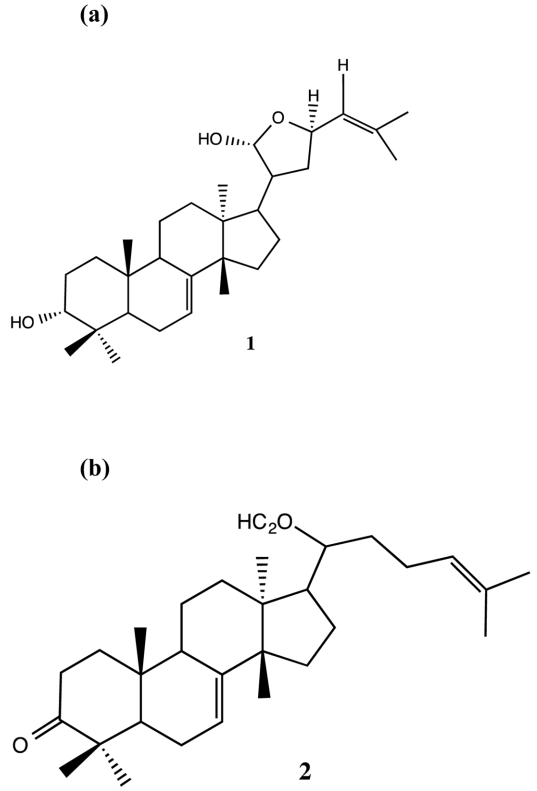

Fig. 1. (a) Chemical structure of flindissol (compound **1**) and (b) 3-oxotirucalla-7,24-dien-21-oic acid (compound **2**).

Experimental

Cell culture – Human breast adenocarcinoma (MCF-7) cell line was courtesy by Prof. Masa-Aki Ikeda of Tokyo Medical and Dental University, Tokyo. MCF-7 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, US) and incubated at 37 ºC in a humidified atmosphere containing 5% CO_2. Cells were treated with the IC_{50}value of each compound. Doxorubicin, an anticancer chemotherapy drug was used as a positive control.

Cell Cycle Analysis – The cells were cultured in 25 cm^2 T-flask to 85% confluence. And then treated with the compound **1** and **2** (13.8 and 27.5 μM, respectively) for 24 h at 37 ºC. The total populations of floating and adherent cells were collected and centrifuged at 900 *g* at 4 ºC for 5 min. The cells were washed with PBS and fixed with 75% cold ethanol. Cells in cold ethanol were centrifuged and washed with PBS to collect the cells' pellet. The cells' pellet were suspended in PBS containing 50 μg/mL Propidium Iodide (PI) (Invitrogen), 0.2 mg/mL RNase A (Sigma) and 0.1% Triton X-100 (Fisher Scientific), and incubated at 37 º for 30 min. Cell cycle profiles were analysed using Guava® easyCyte flow cytometer (Merck Millipore).

mRNA expression studies in MCF-7 cells by quantitative RT-PCR Reverse Transcription – The expression of apoptosis-related genes, such as *PUMA*, *caspase-8* and *caspase-9* were determine by RT-PCR. Total RNA isolation was done using TRIzol® reagent (Invitrogen, USA). Single-stranded cDNA was synthesized from 2 μg total RNA using Omniscript reverse transcriptase (Qiagen). 1 μL reverse transcriptase was required to equal 2 μg of cDNA in 20 μL of reaction volume. The cDNA was determined at 4 μg per reaction, thus reverse transcriptase needed was 2 μL. Briefly, 4 μL of 10×Qiagen one step RT-PCR buffer was added followed by addition of 4 μL of dNTP mix. 0.4 μL of each Oligo-dT primer was added and then 2 μL of Omniscript reverse transcriptase was added. Finally, PCR grade RNAse–free water was added to make total volume of 40 μL and Mixed it gently by vortex and centrifuged in PCR tubes.

Quantitative RT-PCR – RT-qPCR analysis was performed with Quantitect SYBR® Green PCR (Qiagen) kit, in a Corbett Rotor-Gene 6000. 4 μL of RNase-free water was added followed by 7 μL of 2× QuantiTech SYBR Green PCR master mix. 1 μL and 2 μL of primer mix and cDNA was added respectively to a final volume of 14 μL. Each sample was performed in triplicate. Each cycle included an initial denaturation (predenaturation) step of 15 min at 95 ºC. Denaturation step at 95 ºC for 10 sec except *PUMA*, 95 ºC for 40 sec. Annealing at 60 ºC for 30 min for *β-actin*, 55 ºC for 40 min for *PUMA*, 61 ºC for 30 min for *caspase-8* and *caspase-9*. A final extension step was performed in order to complete the PCR reaction, at 72 ºC for 30 min (*β-actin*, *caspase-8* and *caspase-9*) and 73 ºC for 10 min (*PUMA*). 50 amplification cycles were performed for *β-actin* and *PUMA*, and 55 cycles for *caspase-8* and *caspase-9*. The oligonucleotide primer pairs for RT-PCR used in this study were: *PUMA*, 5'-GACC TCAACGCACAGTACGA-3' (forward) and 5'-GAGAT TGTACAGGACCCTCCA-3' (reverse); *caspase-8*, 5'-CAT CCAGTCACTTTGCCAGA-3' (forward) and 5'-GCATC TGTTTCCCCATGTTT-3' (reverse); *caspase-9*, 5'-TTCC CAGGTTTTGTTTCCTG-3' (forward) and 5'-CCTTTC ACCGAAACAGCATT-3' (reverse); *β-actin*, 5'-ATCCG TAAAGACCTCTATGC-3' (forward) and 5'-AACGCAG CTCAGTAACAGTC-3' (reverse). The relative amplification was quantitated through comparison to the expression of *β-actin* as an internal standard. The results were compared with untreated cells using comparative Ct methods ($2^{-\Delta\Delta Ct}$).

Statistical Analysis – The data were analyzed statistically

Table 1. DNA content of MCF-7 cell untreated and treated with different compounds and drug for 24 has determined by PI stained-flow cytometry cell cycle analysis

Samples	Distribution of cells in the cell cycle (%)			
	Sub G_1 phase	G_0/G_1 phase	S phase	G_2/M phase
1	7.7	46	24.4	15.6
2	9.3	41.1	26.5	17.3
Doxorubicin	10.3	41.5	26.3	14.1
Untreated cells	2.7	45.5	30.5	18.9

by comparison of experimental samples of the same treatment conditions as a group with untreated cells and presented as mean standard error (SE). Data were subjected to Student's *t*-test for the determination of level of significance and results were considered significant, if $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results and Discussion

Cell Cycle Analysis – Cell cycle distributions were analyzed by flow cytometry technique. Treated cells with IC_{50} values of compounds **1** (13.8 μM) and **2** (27.5 μM) increased of the cell population at sub G_1 (M4) phase. In this phase, apoptotic cells were quantified as proportion of cells that had a DNA content less than 2*n* due to the detection of fragmented DNA. Table 1 illustrates the changes in the cell cycle distribution of MCF-7 cells treated with compounds **1** and **2** as compared with doxorubicin and untreated cells.

From the results, both compounds induced a significant increase apoptosis at sub G_1 phase in MCF-7 cells after 24 h of treatment. The number of MCF-7 cells population which undergoing apoptosis after the treatment with IC_{50} values of compound **1** (13.8 μM) and **2** (27.5 μM) were found to be 7.7 and 9.3%, respectively (Fig. 2(a) and 2(b)). Whereas, doxorubicin as positive control drug was 10.3% and 2.7% for untreated cells (Fig. 2(c) and 2(d)). At G_0/G_1 phase, both compounds, **1** and **2**, influenced the number of cells to 46 and 41.1% respectively and with a concomitant decrease in the number of cells in S phase to 24.4 and 26.5% respectively. At G_0/G_1 phase, DNA content remained diploid (2*n*) and started to replicate at S phase to become 4*n*. Thus, DNA contents in this phase were in the range of 2*n*-4*n*. Distribution of cells at G_2/M phase for **1** and **2** were 15.6 and 17.3% respectively where DNA content remained 4*n* due to the completion of DNA synthesis and started to decrease to 2*n* after cytokinesis.

mRNA expression studies in MCF-7 cells by quantitative RT-PCR – To elucidate cell death signaling pathway activated by compounds **1** and **2** in MCF-7 cells, RT-qPCR analysis was used to measure the mRNA expressions of pro-apoptotic genes *PUMA*, *caspase-8* and *caspase-9*.

PUMA or p53 upregulated modulator of apoptosis is an essential mediator of p53-dependent and –independent apoptosis.[6] Indeed, the exogenous expression of *PUMA* rapidly induced apoptosis in a variety of human cancer cells.[7,8] From the results of this study, compound **2** showed the higher expression on *PUMA* gene as compared to compound **1** with the fold change of mRNA level of 168.9 and 28.6, respectively. Fig. 3(a) represents the fold changes of mRNA level of *PUMA* normalized to *β-actin*. Thus, with the results of *PUMA* expression, it is proved that both compounds may induce apoptosis.

Caspase-8 is one of the initiator caspases that is closely coupled to pro-apoptotic signals, activated via extrinsic pathway. This pathway activated by engagement of transmembrane receptors by its ligands. Upon ligand binding to death receptor, their cytoplasmic death domain attract adaptor molecule and initiate a caspase cascade. The existence of different receptor/ligand pairs lead to the activation of *caspase-8*. Once *caspase-8* is activated, it cleaves and activates downstream effector caspase including *caspase-3*, -6 and -7[9] which shows the importance of *caspase-8* in apoptosis pathway. The study also analyzed the mRNA expression level in *caspase-8* on MCF-7 cell lines treated with compounds **1** and **2**. The results showed the increased *caspase-8* expression. Fig. 3(b) represents fold change of mRNA level of *caspase-8* normalized to *β-actin*. Compound **2** showed the higher expression on *caspase-8* gene as compared to compound **1** with the fold change of mRNA level of 0.67 and 0.36, respectively. However, both compounds showed lower expression compared to untreated and positive control.

Compound **1** and **2** increased of*caspase-9*expression towards MCF-7 cell lines. Fig. 3(c) represents fold change of mRNA level of *caspase-9* normalized to *β-actin*. Compound **2** showed the higher expression on *caspase-9* gene as compared to compound **1** with the fold change of

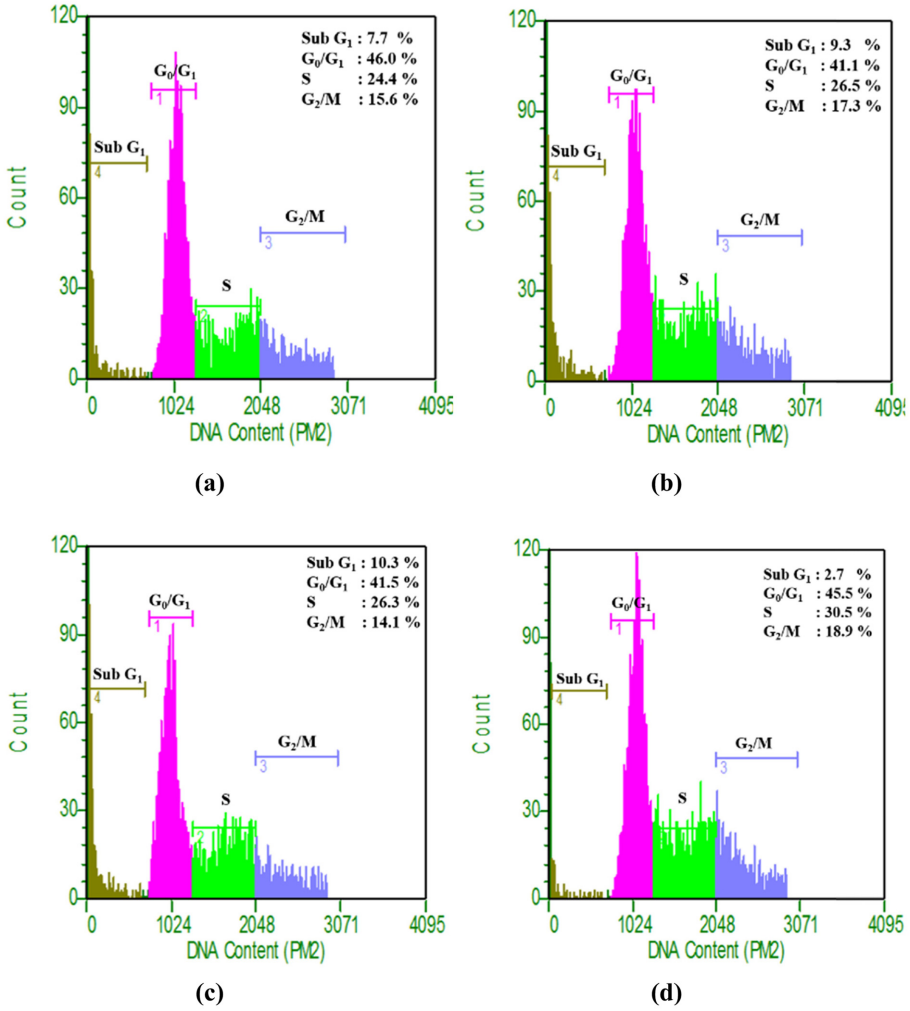

Fig. 2. DNA content frequency histogram of MCF-7 cells treated with IC_{50} value of compound **1**(a), compound **2**(b), doxorubicin (c) for 24 h and untreated cells (d). The cells were stained with PI and analyzed by flow cytometer for DNA content. Markers 1, 2, 3 and 4 represent G_0/G_1, S, G_2/M and sub G_1 (apoptotic cells) phases, respectively.

mRNA level of 38.5 and 23.5, respectively. As mentioned before, *caspase-9* also acts an as initiator caspase which appears to be the first caspase in activation of others. With activation of *caspase-9* by *procaspase-9*, it triggers caspase cascade by activating the effector *caspase-3*, *-6* and *-7*.[8] Hence, it cause morphological and biochemical damages with eventual apoptosis cell death.

The evaluation of gene expression as an evident of apoptosis induction showed that both compounds exhibited high level of all genes including *PUMA, caspase-8* and *caspase-9* expression as normalized to *β-actin* via activation of those genes. Thus, both compounds may induce apoptosis in MCF-7 cells by increasing the *PUMA* and subsequently triggering apoptosis via intrinsic pathway involving *caspase-8* and *caspase-9* genes. However, the involvement of autophagy pathway must be highlighted due to the lack normal *caspase-3* gene in this cell line.

The deficiency of *caspase-3* gene due to a deletion mutation in exon 3 of MCF-7 cell line may not be appropriate to evaluate the effects of compounds on apoptotic induction. Thus, the involvement of autophagy may also constitute in the programmed forms of cells death. Autophagy involves in the etiology of many important human disease including cancer, neurodegenerative disease and metabolic disorders. ATP depletion due to the metabolic stress may cause increase of intracellular calcium and reactive oxygen species (ROS). Cells that do not adapt to these changes may undergo necrotic cell death. However, the activation of protective stress regulators such as adenosine monophosphate-activated protein kinase (AMPK), allow cells to survive these changes. AMPK-dependent phosphorylation activates p53 which leads to

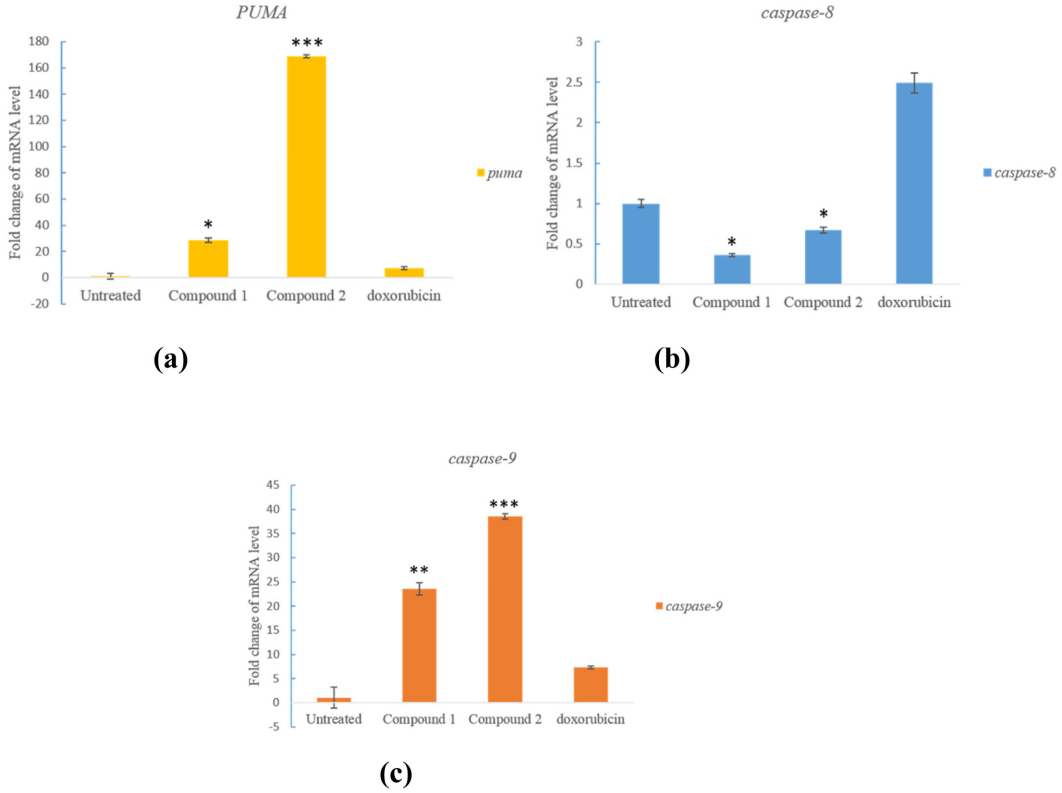

Fig. 3. Effects of compounds **1** and **2** on *PUMA* (a), *caspase-8* (b) and *caspase-9* (c) mRNA level. mRNA level of all genes were normalized to *β-actin* mRNA and fold changes expressed relative to the untreated control. Value shows represent mean ± SE using Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

autophagy or apoptosis through the activation of Bax and Bak, the mitochondrial release cytochrome c and caspase activation. Stress-induced autophagy may promote cell survival or cell death.[10]

Regulation function of p53 is involved in apoptosis and autophagy pathways. Activation of p53 contributes to apoptosis induction mostly via transcription-dependent effects. On the other hand, the involvement of p53 in autophagy may either activate or repress autophagy depending on cellular energy status and the contextual activation of other signaling pathways. Several p53 target genes stimulate autophagy for instance damage-regulated autophagy modulator (DRAM), a p53 target gene encoding a lysosomal protein induces autophagy. The localization of p53 is a key regulator of p53-induced apoptosis and autophagy.[11] With the realization of the lack of *caspase-3* due to a deletion mutation in exon 3 of MCF-7 cell line may not be appropriate to evaluate the effects of compounds on apoptotic induction. Thus, the involvement of autophagy may also constitute in the programmed forms of cells death. Therefore, further researches must be done using these compounds in order to identify the cell death pathway either via apoptosis or autophagy.

Conclusion

It may be concluded that flindissol (**1**) and 3-oxotirucalla-7,24-dien-21-oic-acid (**2**) from *L. scandens* showed anticancer properties in MCF-7 cell line.

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