

Short Communication: Terpenoids isolated from *Lansium domesticum* fruit peel induce apoptosis and cell cycle arrest in T-47D cell lines

KHUSNUL FADHILAH¹, SUBAGUS WAHYUONO^{2,*}, PUJI ASTUTI²

¹Faculty of Pharmacy, Universitas Gadjah Mada. Jl. Sekip Utara, Sleman 55281, Yogyakarta, Indonesia

²Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada. Jl. Sekip Utara, Sleman 55281, Yogyakarta, Indonesia.
Tel./fax.: +62-274-543120, *email: subagusw_fa@ugm.ac.id

Manuscript received: 30 November 2021. Revision accepted: 30 January 2022

Abstract. Fadhilah K, Wahyuono S, Astuti P. 2022. Short Communication: Terpenoids isolated from *Lansium domesticum* fruit peel induce apoptosis and cell cycle arrest in T-47D cell lines. *Biodiversitas* 23: 1167-1171. One of the characteristics of cancer cells is uncontrolled proliferation. This can be caused by aberrant in the proteins that play a role in the process of apoptosis and cell cycle. Lamesticum A and 2-ethyl,3-(1'-hydroxy-2'-menthene) propenal have been isolated from ethyl acetate extract of *L. domesticum* fruit peel had cytotoxicity against T-47D cells. However, the study on how these terpenoids induce cell death is still unknown. Flow cytometry analysis was carried out to evaluate the effect of the compounds in cell cycle modulation and apoptotic induction. The terpenoid compounds induced apoptosis and cell cycle arrest in S phase in a dose dependent manner. Compound 1 synergized the effect of doxorubicin in inducing cell death through mechanism involving the cell cycle. Compound 1 revealed better activity in killing T-47D cells rather than compound 2. Further studies are needed to explore the potential of these compounds in promoting cell death of breast cancer.

Keywords: Apoptosis, breast cancer, cell cycle, cytotoxic, *Lansium domesticum*, T-47D

Abbreviations: DNA: deoxyribonucleic acid, DMSO: Dimethyl Sulfoxide, HEPES: (4-2 (2-hydroxyethyl) -1-piperazineethanesulfonic acid), MTT: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-di-phenyltetrazolium bromide), PBS: Phosphate buffered saline, RPMI: Roswell Park Memorial Institute, T-47D: Breast cancer cell line

INTRODUCTION

Uncontrolled proliferation caused by genomic instability is one of the cancer characteristics. This can be caused by irregularities in the proteins that play a role in the process of apoptosis and cell cycle (Hanahan and Weinberg 2011). Uncontrolled proliferation is caused by deviations in proteins that play a role in the cell cycle. Deviations may occur due to mutations in cell cycle signaling pathways as well as genetic defects in the cell cycle proteins (Tobias and Piotr 2017). The process of apoptosis and the cell cycle are of importance and have become one of the targets in the development of anticancer drugs.

In previous studies, two compounds namely Lamesticum A and 2-ethyl,3-(1'-hydroxy-2'-menthene) propenal from ethyl acetate extract of duku fruit peel (*L. domesticum*) were isolated. Both compounds had cytotoxic activity against T-47D cells with IC₅₀ values of 15.68 and 39.18 µg/mL, respectively (Fadhilah et al. 2020 2021). Lamesticum A was isolated from *L. domesticum* twigs and showed antibacterial activity (Dong et al. 2011). Another onoceranoid-type triterpenoids that isolated from *L. domesticum* leaves showed antimutagenic effect in an in

vitro Ames assay and in vivo micronucleus test (Matsumoto et al. 2018)

In order to examine how these compounds induced cell death, flow cytometric analysis was carried out to evaluate the effect of the compounds on the process of apoptosis and cell cycle of T-47D cells.

MATERIALS AND METHODS

Compound 1 (Lamesticum A) and compound 2 (2-ethyl,3-(1'-hydroxy-2'-menthene) propenal) (Figure 1) were isolated from n-hexane insoluble fraction of the ethyl acetate extract of duku fruit peel from our previous studies (Fadhilah et al. 2020 2021). T-47D cells were collection from Parasitology Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Cell cultures were grown on RPMI (Gibco) medium supplemented with 10% Fetal Bovine Serum (Gibco), 2% Sodium bicarbonate (Gibco), and HEPES (4-2 (2-hydroxyethyl) -1-piperazineethanesulfonic acid) (Invitrogen).

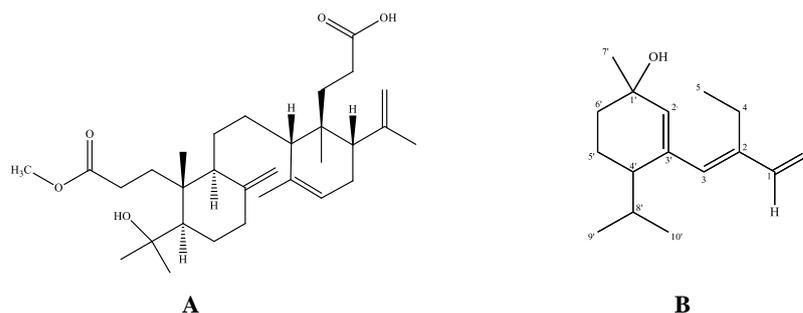


Figure 1. Isolated compounds from *L. domesticum*. A. Lamesticum A, B. 2-ethyl,3-(1'-hydroxy-2'-menthene) propenal

Cell culture

Cells were cultured on 6-wellplate with a total of 10^6 cells/well and incubated at 37°C and 5% CO_2 for 24 hours. After 24 hours, the test compound was added to the cell culture and incubated for another 24 hours. This method was used based on previous studies with modification (Astuti et al. 2012; Pratama et al. 2018). The test compound was dissolved in DMSO (0.05%). In the single compound treatment, the concentrations for compound 1 were $7.84 \mu\text{g/mL}$ ($1/2 \text{ IC}_{50}$) and $15.68 \mu\text{g/mL}$ (IC_{50}) and for compound 2 were $19.6 \mu\text{g/mL}$ ($1/2 \text{ IC}_{50}$) and $39.18 \mu\text{g/mL}$ (IC_{50}). Doxorubicin (Dox) was added at $0.10 \mu\text{g/mL}$ ($1/2 \text{ IC}_{50}$). In the combination test, only compound 1 was tested with concentrations of $7.84 \mu\text{g/mL}$ for compound 1 + $0.10 \mu\text{g/mL}$ of Doxorubicin. Different time of administration was applied in which Doxorubicin was added overnight prior to addition of compound 1 or Doxorubicin was added together with compound 1. Following addition of test compounds, the culture was further incubated for 24 hours. At the end of incubation, the media was removed, and cells were harvested with trypsin ($200 \mu\text{L/well}$). The cell suspension was centrifuged at 2000 rpm for 5 minutes and the cell pellets were washed with warm PBS and again centrifuged at 2000 rpm for 5 minutes. The supernatant was separated, and cell pellets were collected for analysis. Cells were analyzed using FACS (Fluorescence Activated Cell Sorting) Calibur (BD) and software BD FACSDiva 8.0.2.

Apoptotic analysis

For apoptotic analysis, cells were resuspended in $600 \mu\text{L}$ of buffer containing $12 \mu\text{L}$ PI, and $12 \mu\text{L}$ Annexin-V (BD Pharmingen) (Pratama et al. 2018). Cell suspension was homogenized and incubated for 5 minutes. Cells were transferred to a flow-to-tube for analysis. Cell viability, death and apoptosis were evaluated using a flow cytometer.

Cell cycle analysis

The method used was based on previous research (Astuti et al. 2012). In the cell cycle analysis, cells were resuspended in $500 \mu\text{L}$ PBS containing $40 \mu\text{g/mL}$ PI (50x) and RNase A $500 \mu\text{g/mL}$ (Sigma). After staining, the cells were incubated at 37°C for 30 minutes and analyzed with flow cytometer.

RESULTS AND DISCUSSIONS

Attempts to examine how the terpenoid compounds isolated from *L. domesticum* fruit peel kill T-47D cells were conducted by analyzing the apoptotic and cell cycle profiles of the cells following the treatments. Incubating T-47D cells with compound 1 resulted in a slight increase of apoptotic cells. Increased concentration up to $15.68 \mu\text{g/mL}$ induced apoptotic cells until 39.80%. Adding compound 2 at $19.6 \mu\text{g/mL}$ also resulted in a slight increase of apoptotic cells (from 5.66 to 14.33 %). However, unlike compound 1, similar apoptotic profiles were revealed by compound 2 in two different concentrations (Figure 2).

Further study was conducted to examine the effect of the tested compounds on the cell cycle profiles of T-47D cells. In the control untreated cell, 56.6% of cells were in G0/G1 phase, 11.9% were in S phase and 27.1% were in G2/M phase. Adding compound 1 at dose of $7.84 \mu\text{g/mL}$ induced cycle arrest at S phase (22.8%) and G2/M phase (33.6%). The effect, however, was not seen when the compound was added at a higher concentration (Figure 3). It is interesting to note that compound 2 induced cells in S phase (23.1%) at the concentration of $39.18 \mu\text{g/mL}$, which was not observed at a lower concentration of $19.6 \mu\text{g/mL}$. Increase of subG1 population, however, was observed upon addition of compound 2 at higher concentration.

Considering the better ability of compound 1 in inducing apoptosis, further study was conducted to combine the tested compound with doxorubicin. No further increase in the percentage of apoptotic cells was observed when compound 1 was added together with Doxorubicin. However, the total apoptotic cells increased from 18.22% to 22.76% when doxorubicin was added overnight 18 hours prior to compound 1 (Figure 4). Analyzing cell cycle distribution of the cells revealed that a slight increase of cells at S phase with reduced distribution at G2/M phase was observed upon addition of Doxorubicin and compound 1 at the same time. However, reduced cells in G1 phase with more accumulation of cells at S phase (from 8.9% to 12.7%) and a slight increase of cells in sub G1 phase (from 8.3 to 15.7%) were seen, when the compound was added overnight after incubation of T-47D cells with Doxorubicin.

The process of apoptosis and cell cycle arrest is considered as the alternative targets of a chemotherapeutic agent. In cancer cells, there is an imbalance of pro-apoptotic and anti-apoptotic proteins. Overexpression of antiapoptosis and low expression of pro-apoptotic proteins lead to cell growth (Wong 2011). In addition, DNA mutations can cause abnormalities in pro-apoptotic proteins. In the cell cycle, there are regulatory proteins that play a role in confirming genetic material and ensuring the process of cell division properly deviations may also occur due to mutations in the cell cycle signaling pathways as well as genetic damage to the cell cycle proteins.

In this study, two compounds were examined for their ability to induce T-47D cell death. Both compounds seem to interfere with cell cycle progression through modulation in the S phase. The effect appeared to be dose-dependent. The modulation occurred at lower dose for compound 1 and higher dose for compound 2. Both compounds also

increased the number of apoptotic cells. However, significant increase was only seen upon addition of compound 1.

Compound 1 is an onoceranoid-type triterpenoid, while compound 2 belongs to sesquiterpene. The difference in their chemical structures may interfere with the binding of these compounds towards protein targets and thus their consequences on the activities. Compound 1 contains carboxylic acid groups. Some studies reported that there were triterpenoid compounds with carboxylic acid groups with anticancer activity, namely maslinic acid and ursolic acid. Both compounds are pentacyclic triterpenoids with carboxylic acid groups. Maslinic acid has activity to induce apoptosis and cell cycle arrest in G₀/G₁ phase in HT29 colon cancer cells (Reyes-Zurita et al. 2011). Ursolic acid is known to have the ability to activate the p53 pathway for the induction of apoptosis in some cancer cells (Feng and Su 2019).

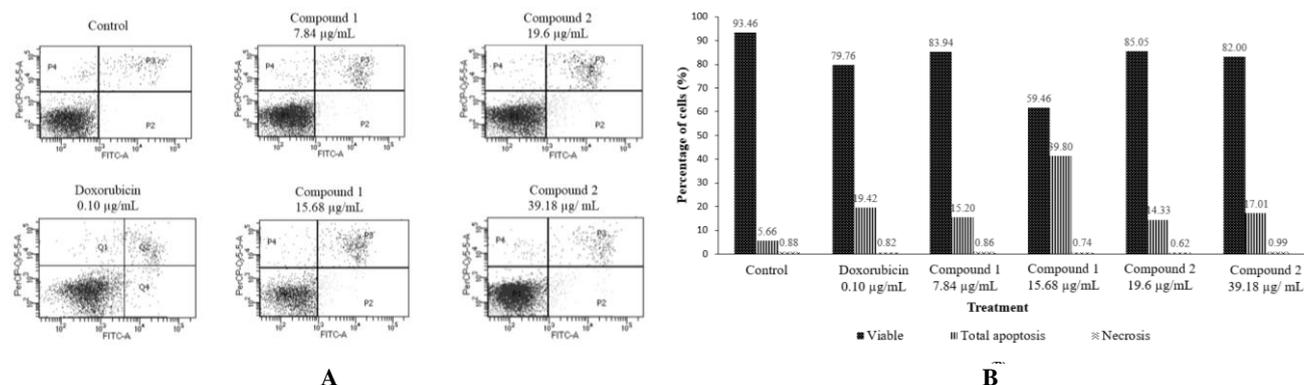


Figure 2. Apoptotic analysis of T-47D cells treated with tested compounds. A. Flow cytometry profiles, B. % distribution of the cells

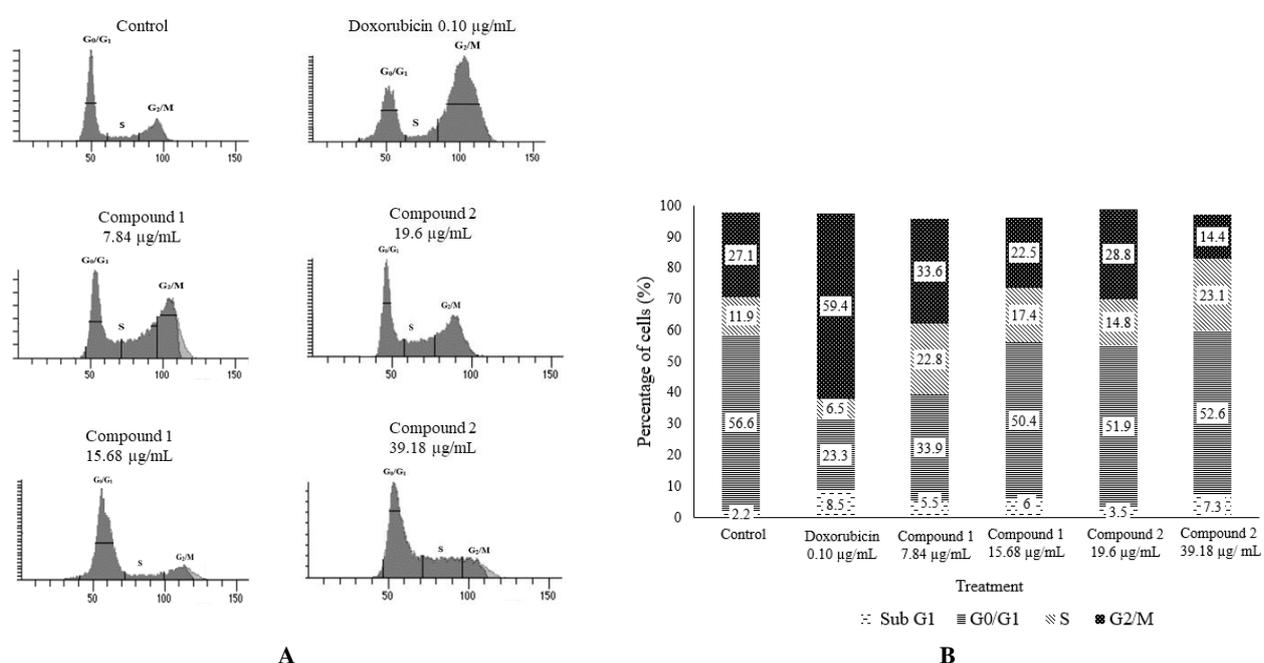


Figure 3. Cell cycle analysis of T47D cells treated with tested compounds. A. Flow cytometry profiles, B. % cell cycle distribution

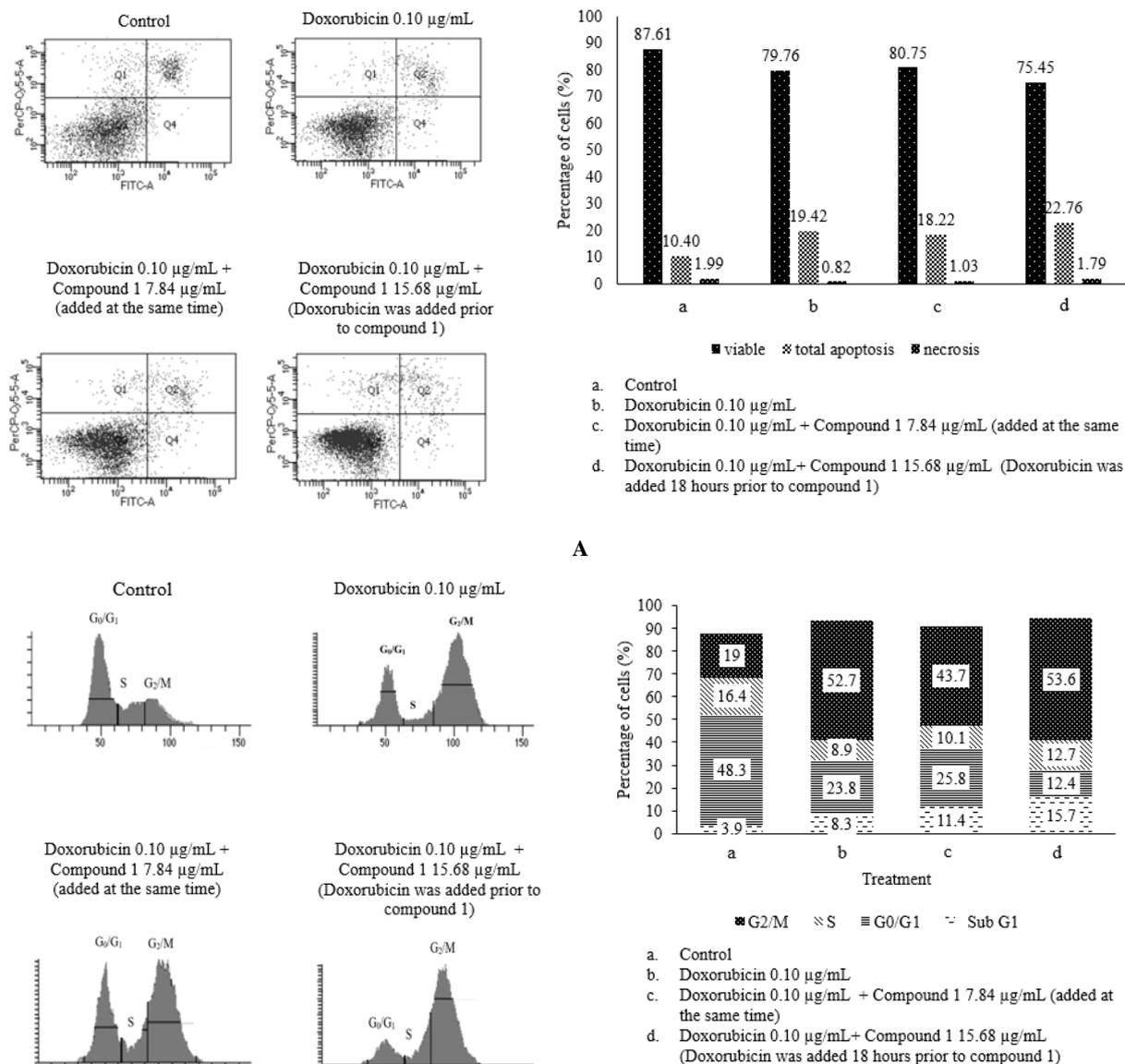


Figure 4. A. Combination effect of compound 1 with Doxorubicin on apoptotic profiles of T47D cells, B. cell cycle distribution of the cells

On the other hand, compound 2 has α,β -unsaturated carbonyl group. Sesquiterpene compounds with alpha-beta unsaturated carbonyl have been widely studied for anticancer activity such as ze rumbone, coronopilin and dehydroleucodine. Zerumbone is known to inhibit the proliferation of HCT116 colon cancer cells by inhibiting TNF- α (Singh et al. 2018). Coronopilin induces G₂/M phase cell cycle arrest in leukemia U937 cells and dehydroleucodine arrests Hela cells in G₁ phase of cell cycle. α,β -unsaturated carbonyl is a reactive site and predicted to play role in proteins that regulate proliferation signaling pathway (Bosco and Golsteyn 2017).

Due to better ability of compound 1 in inducing cell death, further examination was carried out to combine this compound with doxorubicin. Based on previous research, compounds isolated from natural ingredients were known

to be combined with conventional chemotherapeutic agents, generally designated as co-chemotherapy. The combination aimed to increase the effectiveness and reduce the toxic effects of these conventional chemotherapeutic agents. In this study, doxorubicin was used for the chemotherapeutic agent. Several terpenoid compounds had been combined with conventional chemotherapeutic agents including betulinic acid, ursolic acid, vielanins K and P. Betulinic acid was able to induce cancer cell apoptosis when combined with 5-fluorouracil, irinotecan and oxaliplatin (Jung et al. 2007). Ursolic acid was also able to increase the effectiveness of doxorubicin on MCF-7 cells by increasing cellular availability (Zong et al. 2019). Vielanins K and P belonging to sesquiterpenes had been reported to increase the effectiveness of doxorubicin through several signaling pathways. Vielanin K increased

apoptosis of MCF-7 cells through activation of IRE1 α -TRAF2-JNK (Zhang et al. 2020). Meanwhile, vielanin P induced apoptosis of MCF-7 cells by inhibiting MRP-1 expression (Gao et al. 2019). Doxorubicin is a conventional chemotherapeutic agent that inhibited proliferation and caused cell cycle arrest in the G2/M phase (Kim et al. 2009). The G2 phase was the last DNA repair process before cells continue to the division (mitotic) stage. Abrogation of the G2/M phase is one of the alternative targets of cancer treatment therapy. Compounds that are capable in destroying the arrest at G2 phase could prevent the DNA repair process. The DNA repair failure caused genomic instability and apoptosis induction (Bucher and Britten 2008).

Combining compound 1 with Doxorubicin affect G2/M progression when the two compounds were added at the same time. The ability of compound 1 in inducing S phase arrest prevented the accumulation of cells in G2/M phase. The S phase inducing effect was clearly seen when the Doxorubicin was added overnight prior to addition of compound 1. Cells had accumulated at G2/M phase, and further addition of compound 1 did not affect G2/M phase population. Instead, percentage of cells in S phase slightly increased with reduced number of cells in G1 phase and subsequent increase in apoptotic cells. This study indicated that compound 1 induced cell death through different cell cycle mechanism with Doxorubicin and this compound did not act as a G2/M abrogator. Further research is needed regarding the role of compound 1 in S phase of cell cycle.

Since *L. domesticum* belongs to Meliaceae family, some studies reported the effect on apoptosis and cell cycle arrest of extracts and compounds isolated from the Meliaceae family. For instance, aglaodoratin C isolated from *Aglaia odorata* and aglapervericin C obtained from *A. perveridis* induced cell cycle arrest in the G2/M phase (An et al. 2015, 2016). Aphanin, a triterpenoid from *Amoora rohituca* was found to have antiproliferative effect and G0/G1 cell cycle arrest in pancreatic carcinoma cell line (Xu et al. 2019). A rocagalate derivative silvestrol from *A. faveolata* induce apoptosis in prostate cancer (LNCaP) cell line through mitochondrial pathway (Harneti and Supratman 2021). Meliarachin C and 3-O-deacetyl-4'-dimethyl-28-oxosalannin from *Melia azedarach* induced apoptosis by activating the caspase protein (Akihisa et al. 2013). Accordingly, further study is needed to determine the effect of compounds from *L. domesticum* in influencing proteins that play a role in apoptosis and the cell cycle such as caspases and cyclins.

In conclusion, two terpenoids isolated from *L. domesticum* fruit peel induce apoptosis and cell cycle arrest in the S phases. Lamesticumin A (1) exhibited better activity than compound 2 in killing T-47D cells. Combination of lamesticumin A (1) and doxorubicin induces apoptotic and cell cycle arrest in different ways. Doxorubicin was known to arrest in G2/M phase beside Lamesticumin A in S phase. However, further studies are needed to explore the potential of these compounds in promoting cell death of breast cancer.

ACKNOWLEDGEMENTS

Authors highly appreciate to Ministry of Education and Culture of the Republic of Indonesia for research funding (Grant No. 3163/UN1.DITLIT/DIT-LIT/PT/2020). This study is part of Khusnul Fadhillah's Doctoral Program.

REFERENCES

- Akihisa T, Pan X, Nakamura Y, Kikuchi T, Takahashi N, Matsumoto M, Tokuda H. 2013. Limonoids from the fruits of *Melia azedarach* and their cytotoxic activities. *Phytochemistry* 89: 59-70. DOI: 10.1016/j.phytochem.2013.01.015.
- Astuti PD, Utami E, Nugrahani AW, Sudjadi S. 2012. Genistein abrogates G2 arrest induced by curcumin in p53 deficient T47D cells. *J Pharm Sci* 20 (1): 82. DOI: 10.1186/2008-2231-20-82.
- Bosco A, Golsteyn RM. 2017. Emerging anti-mitotic activities and other bioactivities of sesquiterpene compounds upon human cells. *Molecules* 22 (3): 459. DOI:10.3390/molecules22030459.
- Bucher N, Britten CD. 2008. G2 checkpoint abrogation and checkpoint kinase-1 targeting in the treatment of cancer. *British J Cancer* 98 (3): 523-528. DOI: 10.1038/sj.bjc.6604208.
- Dong SH, Zhang CR, Dong L, Wu Y, Yue JM. 2011. Onoceranoid-type triterpenoids from *Lansium domesticum*. *J Nat Prod* 74(5): 1042-1048. DOI: 10.1021/np100943x.
- Fadhilah K, Wahyuono S, Astuti P. 2020. A bioactive compound isolated from Duku (*Lansium domesticum* Corr) fruit peels exhibits cytotoxicity against T47D cell line. *F1000Research* 6 (9): 3. DOI: 10.12688/f1000research.21072.1.
- Fadhilah K, Wahyuono S, Astuti P. 2021. A sesquiterpene aldehyde isolated from ethyl acetate extract of *Lansium domesticum* fruit peel. *Indon J Pharm* 32 (3): 394-398. DOI: 10.22146/ijp.1884.
- Feng XM, Su XL. 2019. Anticancer effect of ursolic acid via mitochondria-dependent pathways (Review). *Oncol Lett* 17 (6): 4761-4767. DOI: 10.3892/ol.2019.10171.
- Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: The next generation. *Cell* 144 (5): 646-674. DOI: 10.1016/j.cell.2011.02.013.
- Harneti D, Supratman U. 2021. Phytochemistry and biological activities of *Aglaia* species. *Phytochemistry* 181: 112540. DOI: 10.1016/j.phytochem.2020.112540.
- Kim HS, Lee YS, Kim DK. 2009. Doxorubicin exerts cytotoxic effects through cell cycle arrest and fas-mediated cell death. *Pharmacology* 84 (5): 300-309. DOI: 10.1159/000245937.
- Matsumoto T, Kitagawa T, Teo S, Anai Y, Ikeda R, Imahori D, Watanabe T. 2018. Structures and antimutagenic effects of onoceranoid-type triterpenoids from the leaves of *Lansium domesticum*. *J Nat Prod* 81 (10): 2187-2194. DOI: 10.1021/acs.jnatprod.8b00341.
- Pratama NP, Wulandari S, Nugroho AE, Fakhruddin N, Astuti P, Sudarsono. 2018. Tylophorine Abrogates G2/M Arrest induced by doxorubicin and promotes increased apoptosis in T47D breast cancer cells. *Asian Pac J Cancer Prev* 19 (11): 3065-3069. DOI: 10.31557/APJCP.2018.19.11.3065.
- Reyes-Zurita FJ, Pachón-Peña G, Lizárraga D, Rufino-Palomares EE, Cascante M, Lupiáñez JA. 2011. The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism. *BMC Cancer* 11: 154. DOI: 10.1186/1471-2407-11-154.
- Singh SP, Nongalleima K, Singh NI, Doley P, Singh CB, Singh TR, Sahoo D. 2018. Zerumbone reduces proliferation of HCT116 colon cancer cells by inhibition of TNF-alpha. *Sci Rep* 8 (1): 4090. DOI: 10.1038/s41598-018-22362-1.
- Tobias O, Piotr S. 2017. Cell cycle proteins as promising targets in cancer therapy. *Nat Rev Cancer* 17 (2): 93-115. DOI: 10.1038/nrc.2016.138.Cell.
- Wong RSY. 2011. Apoptosis in cancer: From pathogenesis to treatment. *J Exp Clin Cancer Res* 30 (1): 1-14. DOI: 10.1186/1756-9966-30-87.
- Xu, WH, Su XM, Wang C, Du F, Liang Q. 2019. The genus *Amoora*: A phytochemical and pharmacological review. *Fitoterapia* 137: 104269. DOI: 10.1016/j.fitote.2019.104269.