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Cytotoxicity of hantap (*Sterculia oblongata* Mast) leaves extracts against breast cancer cell line (T47D): effect on apoptosis and caspase-3 mRNA expression

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ABSTRACT

Hantap (*Sterculia oblongata* Mast) leaves has been traditionally used in Palu, Central Sulawesi to treat breast cancer. However, its scientific evidence is limited. This study aimed to investigate the cytotoxicity of hantap leaf extracts against T47D breast cancer cells. The apoptotic activity of the extracts and its effect on caspase expression were also evaluated. The extracts were prepared by multilevel maceration using n-hexane, methanol, and water. Cytotoxic activity was evaluated by MTT assay. The apoptotic activity was observed by using a fluorescence microscope after acridine orange-propidium iodide (AO/PI) staining, whereas the caspase-3 mRNA expression was examined by using RT-PCR. Among the 3 tested extracts, the methanol extract exhibited the highest cytotoxicity with an IC_{50} value of 85 µg/mL. The methanol extract at concentrations of 42.5 µg/mL (½IC_{50}), 85 µg/mL (IC_{50}), and 170 µg/mL (2IC_{50}) induced 127.25, 85.50, 479.5% of cell apoptosis, respectively. Furthermore, the methanol extract at concentrations of ½IC_{50}, IC_{50}, and 2IC_{50} increased 1.04, 1.43 and 1.69 time higher of caspase-3 mRNA expression. In conclusion, the methanolic extract of hantap leaf exhibits cytotoxicity against T47D breast cancer cells, by inducing apoptosis and increasing caspase-3 mRNA expression.

ABSTRAK

Daun hantap (*Sterculia oblongata* Mast) telah digunakan secara tradisional di Palu, Sulawesi Tengah untuk mengobati kanker payudara. Namun, bukti ilmiahnya terbatas. Penelitian ini bertujuan untuk mengetahui sitotoksisitas ekstrak daun hantap terhadap sel kanker payudara T47D. Aktivitas apoptosis ekstrak dan pengaruhnya terhadap ekspresi caspase-3 mRNA juga dievaluasi. Ekstrak dibuat dengan cara maserasi bertingkat menggunakan n-heksana, metanol, dan air. Aktivitas sitotoksik dievaluasi dengan uji MTT. Aktivitas apoptosis diamati dengan menggunakan mikroskop fluoresensi setelah pewarnaan acridine orange-propidium iodide (AO/PI), sedangkan ekspresi mRNA caspase-3 diperiksa dengan menggunakan RT-PCR. Di antara 3 ekstrak yang diuji, ekstrak metanol menunjukkan sitotoksisitas tertinggi dengan nilai IC_{50} 85 µg/mL. Ekstrak metanol pada konsentrasi 42,5 µg/mL (½IC_{50}), 85 µg/mL (IC_{50}), dan 170 µg/mL (2IC_{50}) masing-masing menginduksi 127,25; 85,50; 479,5% apoptosis sel. Selanjutnya ekstrak metanol pada konsentrasi ½IC_{50}, IC_{50}, dan 2IC_{50} meningkatkan ekspresi caspase-3 mRNA sebesar 1,04; 1,43; dan 1,69 kali lebih tinggi. Simpulan, ekstrak metanol daun hantap menunjukkan sitotoksisitas terhadap sel kanker payudara T47D, dengan menginduksi apoptosis dan meningkatkan ekspresi caspase-3 mRNA.

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INTRODUCTION

Cancer is a disease involving abnormal cell growth with the potential to invade or spread to other parts of the body. Cancer is the leading cause of death worldwide, accounting for an estimated 9.6 million death in 2018.[1] Breast cancer is the most common type of cancer among women worldwide including in Indonesia. In 2022, 2.3 million women diagnosed with breast cancer was reported and 67,000 deaths globally.[2] In Indonesia in 2020, 68,858 (16.6%) new breast cancer cases out of 396,914 new cancer cases with 22,000 of female cancer-related deaths were reported.[3]

Some medicinal plants have been traditionally used by Indonesian people to treat cancer. Hantap (*Sterculia oblongata* Mast) leaves is one of the medicinal plants that used in Palu, Central Sulawesi to treat breast cancer. However, its scientific evidences are still limited. Hantap leaves has been reported to have antioxidants and antifreeradical activities.[4] The phytochemical studies reported that the hantap leaves contains tannin, alkaloids, flavonoids, saponins, oxalate, cyanogenic glycoside, phenol, and lipids.[5] In the previous study reported that methanolic extract of *S. oblongata* Mast leaves exhibits cytotoxic activity against breast cancer cell line (MCF7/HER2).[6]

Dysregulated apoptosis is one of the hallmarks of human cancers including breast cancer.[7] Two major pathways that initiated apoptosis are well known. The extrinsic or death receptor (DR) pathway is activated in response to ligand binding of DRs superfamily members, resulting in activation of capase-8 and caspase-3. The intrinsic pathway is activated by mitochondrial release of cytochrome c, leading to formation of Apaf-1 and cytochrome c, subsequently activating caspase-9 and caspase-3.[7,8] It was reported that caspase-3 can serve as a terminal splicing enzyme in apoptosis and participate in the mechanism of anticancer.[9]

This study aimed to evaluate the cytotoxic activity of leaves extracts against the T47D breast cancer cell line. In addition, the effects of these extracts on apoptosis and caspase-3 expression were also investigated.

MATERIALS AND METHODS

Design of study

This was an experimental laboratory using the post-test only control group design. Cytotoxic activity of the extracts were evaluates by using the MTT assay. Apoptosis examination of T47D was performed after 24 h incubation with tested extract using doxorubicin as a positive control. Observation of apoptosis was performed by using a fluorescent microscope after acridine orange-propidium iodide (AO/PI) staining. The caspase-3 mRNA expression was examined using RT-PCR. The protocol was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (UGM)/Dr. General Hospital, Sardjito, Yogyakarta (ref. No. KE/FK/1213/EC, November 5, 2020).

Extract preparation

Hantap plant was obtained from Palu, Central Sulawesi and was identified at the Herbarium of Universitas Tadulako, Palu. The leaves of hantap were cleaned and dried at room temperature for 24 h. The dried leaves obtained were weighed, ground into simplicia powder, and stored at 4°C. Extract was then prepared by multiple maceration by using n-hexane, methanol, and water at the Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, UGM, Yogyakarta. The powdered leaves were macerated in

n-hexane with intermittent shaking for three days and filtered. The remaining residue was further macerated two time by using n-hexane. All the n-hexane filtrates were pooled and evaporated by using rotary evaporator at 30-40 ^{o}C to obtain dried chloroformic extract. The remaining residue was then sequently macerated by using methanol and water similar to the procedure conducted for the n-hexane maceration. The yield of each extract was weighed and stored at 4 °C until analyzed.

Preparation of T47D cell growth media

The T47D cell growth and cytotoxicity test was conducted at the Department of Parasitology, Faculty of Medicine, Public Health and Nursing, UGM, Yogyakarta. The cell growth media were prepared by combining 10 mL FBS, 0.5mL fungison, 2 mL penstrep, 1 mL mycoXpert (50x) Mycoplasma Removal Reagent, and 0.1% trypsin. Subsequently, 87.5% of the stock media was added. The resulting solution was filtered through a sterile 0.2 µm polyethylene sulfone filter, transferred to a closed sterile bottle, and stored at 4°C.

Cytotoxicity test

Cytotoxicity test of hantap leaf extract against T47D cancer cells was conducted by MTT assay. Each extract was pre-solubilized in DMSO at 37 ^{o}C to obtain a stock solution. Serial dilution of the tested extracts at a concentration of 400 to 12.5 µg/mL was prepared from the stock solution with a final concentration of DMSO lower than 1%. 100 µL of the T47D cell lines suspension in a medium containing 2 x 10^4 cells were filled in a 96-well microplate in triplicate and incubated at 37 ^{o}C in a CO_2 incubator for 24 h. The cells were then incubated with the serial dilution of the tested extracts for 24 h. The cell lines suspension without the tested extract was used as the negative control and doxorubicin as the positive control. After 24 h incubation period, supernatants were removed from the wells and 25 µL of the MTT solution (2 mg/mL) in PBS was added to each well and incubated at 37 ^{o}C for 4 h. The formed formazan crystals, directly correlated to the number of viable cells in the culture, were solubilized by the addition of 125 µL of DMSO to each well. The plates were placed on a shaker for 15 min and the optical density was determined at λ 595 nm on an ELISA reader. The cytotoxic activity was expressed by the 50% inhibitory concentration (IC_{50}) which is defined as the extract concentration needed for a 50% reduction of cell viability. The IC_{50} value was determined using a non-linear regression curve and presented as mean ± standard error of the mean (SEM).

Cell apoptosis assay

For the most active extract, methanolic extract, its effect on apoptotic cell was evaluated by using a fluorescent microscope after AO/PI staining at the Department of Parasitology, Faculty of Medicine, Public Health and Nursing, UGM. After seeding in 24 well-plate on a glass slide and overnight incubation at 37° C, the T47D cells line was treated with 3 different concentrations of the tested extracts (½IC_{50}; IC_{50} and 2IC_{50}) and incubated at 37 ^{o}C for 24 h. Doxorubicin in the concentration of IC_{50} was used as a positive control. Following incubation, the cells were harvested and washed twice with PBS. Glass slides were subsequently stained with 10 µg/mL of AO/PI for 5 min in the dark at 37˚C, followed by washing with PBS 3 times. Stained cells were observed under a fluorescence microscope (excitation wavelength, 488 nm; magnification, x200). The apoptotic cell in 100 cells was counted within 20 min by using a fluorescent microscope. Viable cells showed green fluorescent,

whemRNA reas apoptotic cells showed orange fluorescent. The assay was repeated 3 times.

Caspase-3 mRNA expression assay

The caspase-3 mRNA expression test wasperformedbyRNAextractionusingthe Favorgen kit. Next, reverse transcriptase was performed, followed by the optimization of RT-PCR caspase-3 and RT-PCR GAPDH caspase-3. Caspase-3 primer sequences were as follows: Forward 5'-CCGACTTCCTCTCTGCTTACTC-3'; Reverse 5'-CGTACAGTTTCAGCATGGC-3'. The GAPDH primer, serving as a housekeeping gene, had the following sequences: Forward 5'-TCCCGTTGATGACCAGCTTC-3'; Reverse 5'-GTTACCAGGGCTGCCTTCTC-3'. Relative expression analysis of mRNA was conducted according to the Livak method and the $2^{-\Delta\Delta CT}$ algorithm formula, with GAPDH serving as the reference gene.

Statistic analysis

Data analysis was performed using Microsoft Excel and SPSS Statistics Ver-26 for Windows (IBM, USA). Quantitative data were displayed as mean ± standard error of the mean (SEM). One-way Anova was applied to parametric data and continued with Tamhane's post hoc test to determine significant differences between groups. A p value <0.05 was considered a significant.

RESULTS

Cytotoxic test

The T47D cell's growth after 24 h incubation with hantap leaf extracts or doxorubicin is presented in FIGURE 1. Based on these curves the IC_{50} was determined. Among 3 extracts tested, the methanolic extract exhibited the most active extract against T47D cells with an IC_{50} of 85 µg/mL. The IC_{50} of the water and hexanic extracts were 225 µg/mL, and 1934 µg/mL, respectively. The IC_{50} of doxorubicin as a positive control was 0.393 µg/mL.

Apoptosis examination

For the most active extract i.e. methanolic extract, its effect on the T47D cells apoptosis was investigated. FIGURE 2 shows the fluorescent microscopic examination of T47D cells after 24 h incubation with the methanolic extract at various concentration (½IC_{50}; IC_{50} and 2IC_{50}), and staining with AO/PI. Green-stained T47D cells represent viable cells (VL), whereas red staining represents apoptotic cells (AL).

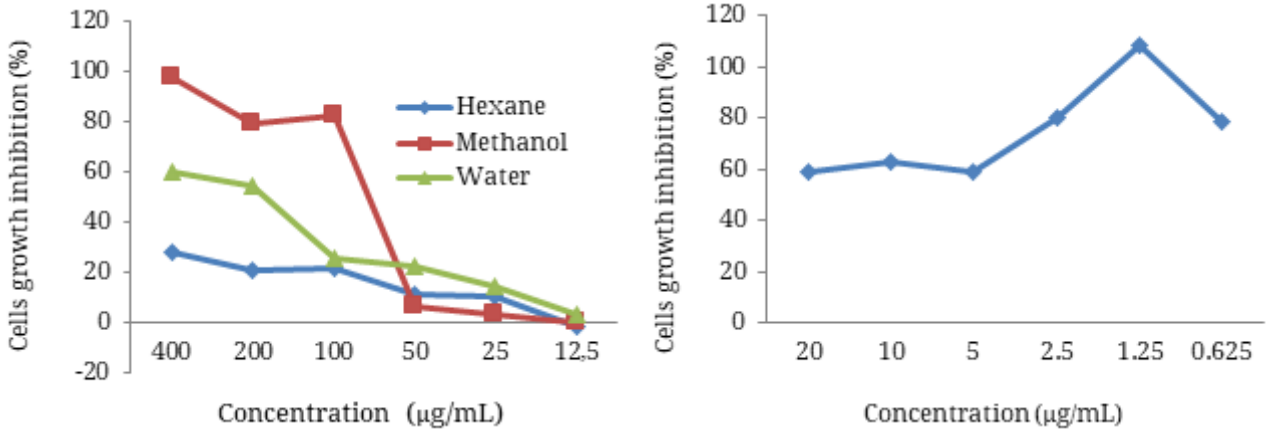

FIGURE 1. Inhibition of T47D cell growth after 24 h of incubation with A) different concentrations of hantap leaves extracts, and B) doxorubicin.

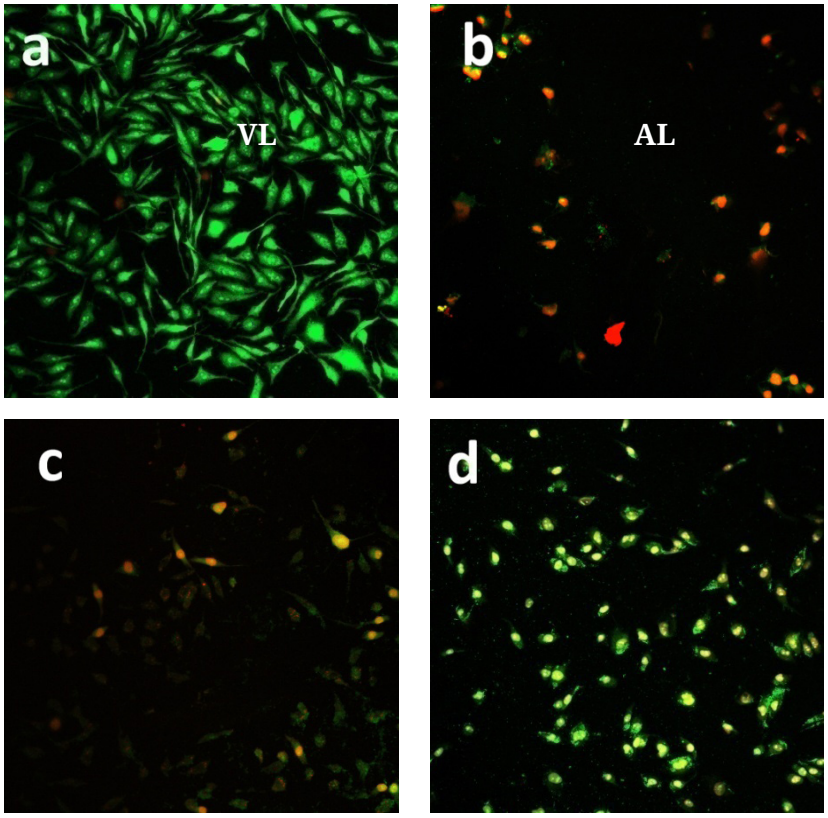

FIGURE 2. The fluorescence microscopic examination of apoptotic T47D cancer cells after 24 h incubation with methanolic extract of *S. oblongata* Mast. a) Control cells, b) methanolic extract with $2IC_{50}$, c) IC_{50}, and d) $½IC_{50}$. Live cells (VL/Green) and apoptotic cells (AL/Red)

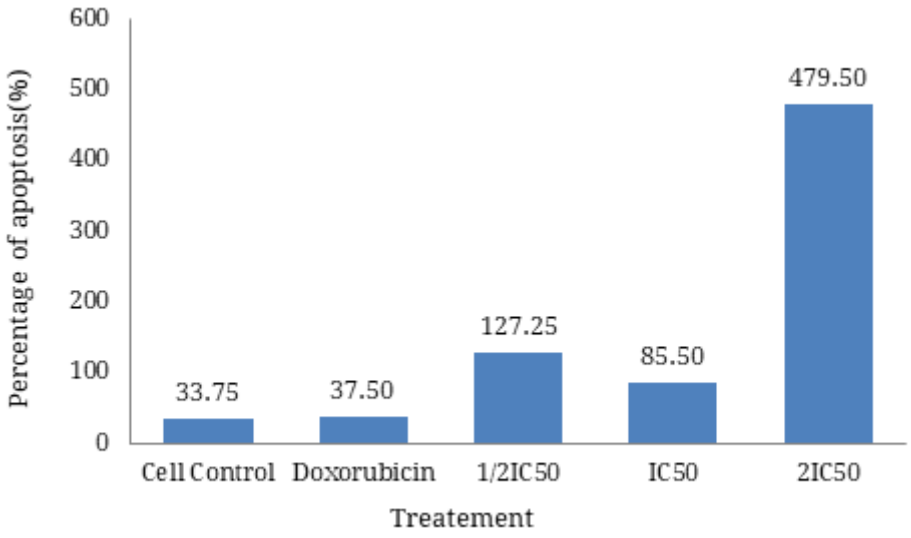

FIGURE 3. Percentage of T47D apoptotic cells after 24 h incubation with methanolic extract of hantap leaves at different concentrations.

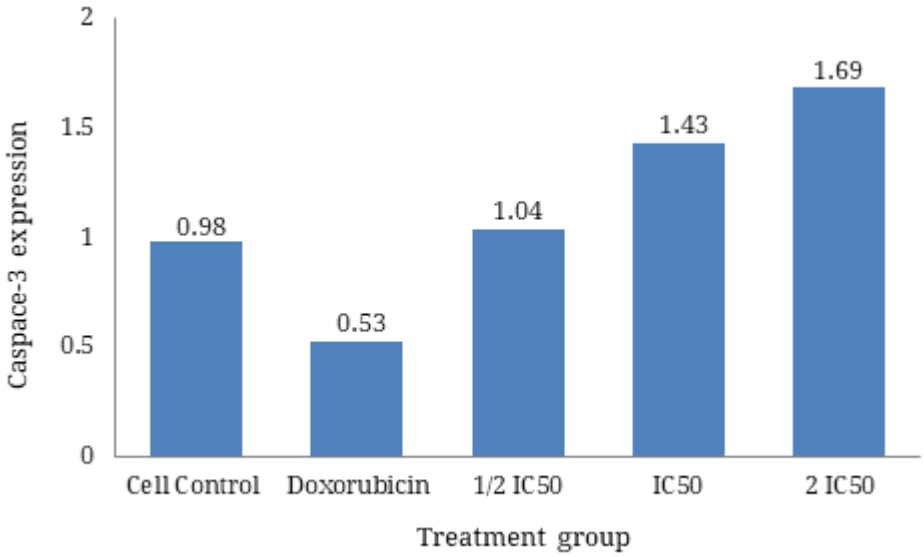

FIGURE 4. Expression of caspase-3 mRNA of the T47D cancer cell line after 24 h incubation with methanolic extract of hantap leaf and doxorubicin.

FIGURE 3 shows the effect of methanolic extract of hantap leaf at different concentrations on apoptosis of the T47D cancer cells. The percentage of T47D cancer cells undergoing apoptosis after 24 h incubation with methanolic extract at concentrations of ½IC_{50} (127.25%), IC_{50} (85.5%), and 2IC_{50} (479.5%) were significantly higher ($p<0.05$) than doxorubicin as positive control (37.5%) and negative control (33.75%). No significantly different in apoptosis of the T47D cancer cells after doxorubicin and negative control was observed ($p> 0.05$).

Caspase-3 mRNA expression examination

The effect of methanolic extract on caspase-3 mRNA expression was also evaluated. FIGURE 3 shows the effect of methanolic extract of hantap leaf at different concentrations on capspase-3 mRNA expression of the T47D cancer cells. The percentage of the caspase-3 mRNA expression of T47D cancer cells after 24 h incubation with methanolic extract at concentrations of ½IC_{50} (1.04), IC_{50} (1.43), and 2IC_{50} (1.69) were significantly higher ($p< 0.05$) than that doxorubicin as positive control (0.53). The caspase-3 mRNA expression after 24 incubation with doxorubicin was significantly lower than that the cell control (0.98; $p<0.05$).

DISCUSSION

In this study, three hantap leaves extracts, namely methanol, n-hexane, and water extracts were evaluated their cytotoxic activity against the T47D cancer cell lines. The methanolic extract exhibited the most active extract against T47D cell lines with an IC_{50} of 85.5 µg/mL. The American National Cancer Institute (NCI) classifies cytotoxic activity of an extract into four groups i.e. high activity if the IC_{50} value is ≤20 µg/mL, moderate activity if the IC_{50} value in the range of 21–200µg/mL, weak activity if the IC_{50} is in the range of 201–500µg/mL, and lacking activity if IC_{50} value is >500 µg/mL.[10] Based on this classification, the methanolic extract is considered to have moderate cytotoxic activity. Cytotoxic activity of the hantap leaf extracts against

cancer cell line have been reported in the previous study. Yahya *et al.*[6] reported that methanolic extract of hantap leaf (*S. oblongata* Mast) has moderate cytotoxic activity against MCF7/HER2 breast cancer cell line with an IC_{50} value of 91.25 µg/mL.

This study also demonstrated that the methanolic extract of hantap leaves can induce apoptosis in T47D breast cancer cell lines. This result is consistent with a previous study conducted by Yahya *et al.*[6] who reported that the methanolic extract of hantap leaves can induce cell apoptosis in MCF7/HER2 breast cancer cell lines. Furthermore, this study showed that the methanolic extract of hantap leaves enhances caspase-3 expression in T47D breast cancer cell lines in dos dependent manner (FIGURE 4).

The genus *Sterculia* and the related genera have been reported for their antioxidant, antiinflammatory, antimicrobial and cytotoxic activities.[4,11,12] The *Sterculia* genus contains mainly flavonoids, whereas terpenoids, phenolic acids, phenylpropanoids, alkaloids, and other types of compounds including sugars, fatty acids, lignans and lignins are of less distribution.[12,13] Flavonoids of the hantap are believed to be responsible for their strong antioxidant and anticancer activities. Many studies reported the potential anticancer activity of flavonoids promoting apoptosis in cancer cells.[14]

Apoptosis is a cellular mechanism that permanently eliminates cell damage without inducing inflammation.[15-17] It is a homeostatic mechanism, maintaining a balance between cell death and cell divisi onto up hold an appropriate cell count in the body.[18] The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. It is reported that there are two main apoptotic pathways: the extrinsic pathway and the intrinsic pathway. The extrinsic signaling pathway (death receptor-dependent pathway) is initiated by the interaction of exposed death receptors of the cell surface belonging to the tumor necrosis factor receptor (TNFR) superfamily with their respective TNF family ligands such as TNFR1-TNFα, FAS (CD95, APO1-FasL), TRAILR1 (death receptor 4; DR4)-TRAIL, TRAILR2 (death receptor 5; DR5)-TRAIL.[16,19,20] The intrinsic signaling pathway (mitochondrial pathway) is initiated by several stimuli that act on multiple targets within the cell such as irreparable genetic damage, hypoxia, high cytosolic Ca^{+} concentration, dan severe oxidative stress.[16,19,20]

The different classes of flavonoids such as flavones, flavonols, isoflavones, chalcones, prenylflavonoids interfere with multiple signal transduction pathways leading to limit proliferation, angiogenesis, and metastasis or increase apoptosis. In apoptosis process, flavonoids interfere with both the extrinsic and the intrinsic pathways to induce apoptotic cell death in human cell cancer.[20,21] Flavonoids induce extrinsic apoptotic pathway by upregulated, FAS, FASL, DR4 and DR5 and down regulated key anti-apototic proteins such as cellular FLICE-inhibitory protein (c-FLIP), X-linked inhibitor of apoptosis protein (XIAP), or survivin.[20,22] Furthermore, flavonoids induce intrinsic apoptotic pathway by induced caspase-dependent apoptosis, or over produced reactive oxygen species (ROS).[22]

Overall, methanolic extract of hantap leaves (*S. oblogata* Mast) had moderate cytotoxic activity against T47D breast cancer cell lines. This is a potential medicinal plants for treatment of cancer. Further study is needed to separate and isolate of active compounds for the development of anticancer.

CONCLUSION

In conclusion, among hexanic and water extracts of hantap leaves (*S. oblongata* Mast) tested, the methanolic

extract exhibited the most cytotoxic against T47D breast cancer cell line, with moderate activity (IC_{50} value of 85 µg/mL). This methanolic extract induces the cancer cell apoptosis by increased caspase-3 mRNA expression. Further study will be performed to isolate active compounds as anticancer from *S. oblongata* Mast.

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