

## RESEARCH ARTICLE

### Antiproliferative properties of tiliroside from *Guazuma ulmifolia* lamk on T47D and MCF7 cancer cell lines

Muhammad Da'i, Erindyah Retno Wikantyasning, Arifah Sri Wahyuni, Ika Trisharyanti Dian Kusumawati, Azis Saifudin, Andi Suhendi

Department of Pharmacy, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, Kartasura, Surakarta, Indonesia

Correspondence to: Muhammad Da'i, E-mail: Muhammad.Dai@ums.ac.id

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#### ABSTRACT

**Background:** Previous research showed that the extract of guazuma leaves had cytotoxic activity on various cancer cell lines. One of the chemical markers of *Guazuma ulmifolia* Lamk. is tiliroside. **Aims and Objectives:** The aim and objective of this study was to determine the cytotoxic effects of tiliroside on T47D and MCF7 and their mechanisms. **Materials and Methods:** Tiliroside (was isolated by laboratory Biological Pharmacy of Universitas Muhammadiyah Surakarta, was confirmed by using liquid chromatography-tandem mass spectrometry/MS). The antiproliferative activity was analyzed by flow cytometry and immunocytochemistry methods. **Results:** Tiliroside has cytotoxic activity with inhibitory concentration 50% on T47D and MCF7 which was 67.79 and 100.00 µg/ml, respectively. Based on the immunocytochemistry, tiliroside on T47D cell lines induced apoptosis which is mediated by the activation of Caspases 8 and 9 and decreased expression of Bcl-2 protein without interference with gen protein 53 (p53). In line with the previous one, tiliroside on MCF7 cell lines induced apoptosis through extrinsic ways due to the underexpression of p53 and Caspase 9 and increase in the expression of Bcl-2 and Caspase 8. **Conclusions:** The antiproliferative effects of tiliroside are higher on T47D than MCF7 cancers cell lines. Based on molecular analysis, antiproliferative mechanism was by apoptosis through extrinsic pathways.

**KEY WORDS:** Tiliroside; Antiproliferative; Apoptosis; MCF7; T47D

#### INTRODUCTION

The cases of cancers are increasing year by year, and the mortality caused by cancers has a high percentage.<sup>[1]</sup> Two types of cancers which have a big contribution on mortality are breast and cervix cancers. Data from the National Cancer Institute have revealed that about 40,000 women died by breast cancers.<sup>[2]</sup> In general, the types of cancer therapies are surgery, chemotherapy, and radiation,<sup>[3]</sup> even though these therapies have limitations that they fail to treat the cancers.

Due to lack of cancer therapy and high side effects by radiation and chemotherapy, we need to find new safe chemotherapy agent including herbs or plants. This research was conducted to elucidate the potency of *Guazuma ulmifolia* lamk as antiproliferative agent. Previous research had stated that *G. ulmifolia* Lamk has various biological activities such as antihypertensive,<sup>[4]</sup> antimicrobial,<sup>[5]</sup> antiulcer,<sup>[6]</sup> antioxidant,<sup>[7]</sup> and cytotoxic on MCF7 cancer cell lines<sup>[8]</sup> and T47D.<sup>[9]</sup> Biological activities of *G. ulmifolia* Lamk due to tiliroside content. Esteves-Souza et al.<sup>[10]</sup> have stated that tiliroside has cytotoxic activity on leukemia cell lines and research by Tomczyk et al.<sup>[11]</sup> showed the cytotoxic activity of tiliroside on MCF7 cancer cell lines.

Tiliroside induce apoptosis was reported by inhibit Nuclear factor-κB, transcription factor of anti-apoptotic protein.<sup>[12]</sup> Programed cell death of cancer cell lines is also

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called apoptosis. There are two ways of apoptosis, namely extrinsic and intrinsic pathways. Apoptosis mechanism could be confirmed by immunocytochemistry which detects specific protein expression or antigen in cell culture. This method is performed by direct and indirect measurements.<sup>[13]</sup> In this research, we analyzed the antiproliferative effects of tiliroside by immunocytochemistry with direct measurement.

## MATERIALS AND METHODS

T47D and MCF7 cells were provided by Professor Masashi Kawaichi (Nara Institute Science and Technology, Japan). Dulbecco's Modified Eagle's medium - high glucose D6655 SIGMA - culture medium for MCF7 cells, medium supplemented with 5% fetal bovine serum (F6178, SIGMA), Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Catalog number: 31800022, culture medium for T47D cells), 100 U/ml penicillin and 100 µg/ml streptomycin (P4333 SIGMA), Trypsin-EDTA solution (T4049 SIGMA), sodium dodecyl sulfate (SDS), (Invitrogen, catalog number: 28312), monoclonal anti-Caspase 9 clone CAS9-purified mouse immunoglobulin product number C 4356, monoclonal anti-Caspase 8 CAS8, monoclonal antibody produced in mouse C4106 SIGMA, monoclonal anti-Bcl-2 antibody produced in mouse B3170 SIGMA, monoclonal anti-protein 53 (p53) antibody produced in mouse clone DO-7, purified from hybridoma cell culture P8999 SIGMA, Starr Trek Universal HRP Detection Kit (Catalog Number: STUHRP700 H, consists of (a) Trekkie Universal Link (STU700H) 1×25 ml, (b) TrekAvidin-HRP (STHRP700H) 1×25 ml 3. betazoid 3,3'-diaminobenzidine (DAB) chromogen (BDB900C) 1×1 ml, (c) betazoid DAB substrate buffer (DS900H) 1×25 ml were used. Tiliroside was isolated from *Guazuma unifolia* extract by chromatography method. Isolate was confirmed by liquid chromatography-tandem mass spectrometry/MS (isolation and identification in pharmaceutical chemistry laboratory of the Faculty of Pharmacy, Universitas Muhammadiyah Surakarta).

### Cytotoxicity test

Cells were harvested by trypsinization and subsequently collected in a 15 ml conical tube and centrifuged at 1000 rpm for 5-10 min. The supernatant was discarded, and the cells were washed once with 1 ml PBS and spun at 1000 rpm for 5 min. The supernatant was again discarded, and the culture medium with FBS 10% was added up to 1 ml. Cells were seeded onto 96-well plate (15,000 cells in each well) and incubated for 24 h (incubator CO<sub>2</sub> 5%, with temperature 37°C). The samples (tiliroside and doxorubicin) were added with the final concentration as indicated (100, 50, 25, and 12.5 µg/ml). The cells were incubated for 24 h (incubator CO<sub>2</sub> 5%, with temperature 37°C). The medium was discarded and replaced with the new culture medium with FBS 10%, added with MTT (5 mg/ml) and followed by 4 h incubation.

The formazan was diluted in 10% SDS HCl (0.1%). The result was measured using ELISA reader (10013301i, iMark Microplate Reader at 595 nm).

### Flow cytometry

Tube containing cells discharged by micropipette and the sediment (cells) was remained. A volume of 100 µL of flow cytometry reagent was added to each tube and then vortexed. Suspension was incubated for about 10 min in dark. Cell suspension (T47D, MCF7) was transferred to flow cytotube and 350 µL of PBS was added in each tube. To know the percentage of viable cells after treatments, flow cytotube was read by flow cytometer.

### Immunocytochemistry

Amount of cell about  $5 \times 10^5$  with 2000 µL of RPMI was cultured on to cover slip of 6-well plate and then incubated for a night. Cells were washed, and RPMI was replaced with fresh culture media. Cells were treated by tiliroside of 30 µg/ml and incubated for 24 h. Cells were treated by doxorubicin 14 µg/ml and control without treatment conducted as previous steps. Cells were incubated (incubator CO<sub>2</sub> 5%, with temperature 37°C) for 15 h, afterward washed by PBS, and later on, the cells were fixed using 300 µL of methanol for 10 min. Cover slips were washed using PBS twice and H<sub>2</sub>O<sub>2</sub> 10% was added, later followed by 100 µL blocking serum. Thereafter, cells were treated by 50 µL primary antibody. The primary antibodies were p53 (monoclonal mouse anti-human p53 protein clone DO-7), Bcl-2 (monoclonal mouse anti-human Bcl-2 oncoprotein clone 124), Caspase 8 (monoclonal mouse anti-human Caspase 8 clone Caspase 8), and Caspase 9 (monoclonal mouse anti-human Caspase 9 clone Caspase 9). Primary and secondary antibodies were diluted in PBS. The secondary antibody used was TrekAvidin-HRP (streptavidin-horseradish peroxidase) and chromogenic reagent was DAB. All these steps were adhered to the instructions on CCRC UGM ([www.crc.farmasi.ugm.ac.id](http://www.crc.farmasi.ugm.ac.id)) with modifications.

## RESULTS

### Cytotoxic Activity Assessment of Tiliroside on T47D and MCF-7 Cell Lines

Determination of cytotoxic activity of tiliroside was performed by MTT assay method. MTT is an accurate, fast, and sensitive method compared with trypan blue method. In this method, MTT will be absorbed into mitochondria, then it will be reduced by reductase enzymes, and formazan crystals are formed.<sup>[14]</sup> The concentration of formazan crystal was measured by ELISA reader at 595 nm. The concentration of formazan was calculated to obtain the inhibitory concentration 50% (IC<sub>50</sub>) value (Tables 1 and 2). The result showed that tiliroside has cytotoxic activities on T47D and MCF7 with IC<sub>50</sub> values of 67.79 and 112.77 µg/ml, respectively. Based

**Table 1:** Effect of tiliroside and doxorubicin on T47D and MCF7 cell lines

Treatment	Percentage of viable cells		Beginning apoptosis		End apoptosis		Necrosis	
	MCF7	T47D	MCF7	T47D	MCF7	T47D	MCF7	T47D
Tiliroside	90.96	86.45	1.07	6.66	3.18	4.93	4.8	2.05
Negative control	92.86	77.32	1.66	17.64	2.44	4.02	3.08	1.15
Doxorubicin	0.52	6.69	0.00	28.16	3.45	13.89	96.05	51.48

on the study by Aboul-Enim et al.,<sup>[15]</sup> these activities were classified as weak activity, but other researchers classified them as moderate.<sup>[16]</sup> Based on IC<sub>50</sub> value, it was observed that tiliroside has a low cytotoxic activity on MCF7 cell lines.

MCF-7 cancer cell lines are characterized by resistance chemotherapeutic agents,<sup>[17,18]</sup> responsive estrogen receptors (ER+), overexpression of Bcl-2,<sup>[19,20]</sup> and with no expression of Caspase 3.<sup>[21,22]</sup> Stimulation of ER could increase P-gp concentration.

To evaluate the effect of tiliroside on *G. ulmifolia* Lamk on the growth of T47D and MCF7 human breast cancers cells, the cell viability was quantified using MTT assay. The tiliroside showed more toxic effect on T47D than on MCF7 cancer cell lines. As shown in Figure 1, on concentration of tiliroside 100 µg/ml the viability cell T47D was lower than MCF7. In general, the data described that tiliroside has lower activity on T47D and MCF7 than doxorubicin, even though doxorubicin is more sensitive on T47D than MCF7. This study gave the same result of the study by Zampieri et al.<sup>[23]</sup> that showed the resistance of MCF7 to doxorubicin.

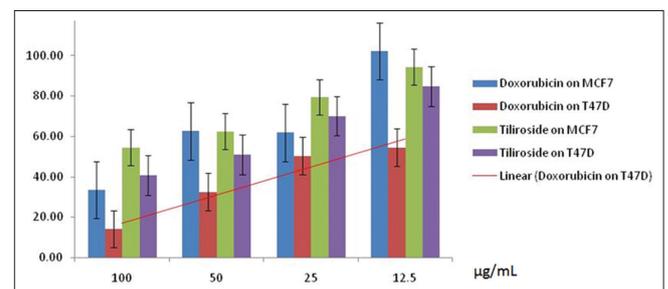
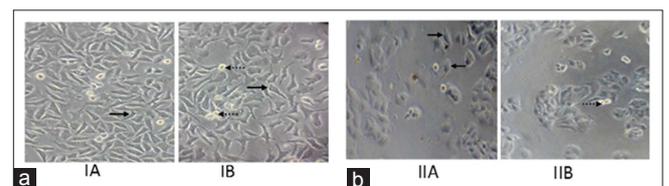
By the morphological properties of T47D cell using microscope, it was found that there is a difference between alive and dead cells (Figure 2). Alive T47D cells attached to the bottom of the well and dead cells were presented as floating round cell. There is a morphological difference between T47D and MCF7. Alive MCF7 cells attached to the bottom of the well and dead cells present as floating round cell.

Previous data showed the cytotoxic activity of tiliroside on T47D and MCF7 cancer cell lines to know how the compound inhibits the proliferation of cancer cell lines, which is conducted by flow cytometry method. This method was based on different color fluorescents formed as the response of cancer cell lines to the test compound. Data obtained were percentage of viability cell (R1), beginning apoptosis (R2), end apoptosis (R3), and necrosis (R4). There is a different condition on each assay. In alive cancer cell lines, phosphatidylserine (PS) positions on cytoplasmic surface; however, on apoptotic process, rapid changes of phospholipid lead to dismantling of PS from inside to surface cell.<sup>[24]</sup> To contrast these conditions, annexin V-FITC and propidium iodide reagent were used. These reagents could distinguish R1, R2, R3, and R4 (Figures 3 and 4) based on the difference of permeability of membrane PS. The color formed for R1, R2, R3, and R4 were green, yellow, pink, and red, respectively. Formed color was due to emission of

**Table 2:** Expression of p53, Bcl-2, caspase 8, and caspase 9 by treatments

Groups	p53	Bcl-2	Caspase 8	Caspase 9
Tiliroside	***	*	***	***
Doksorubisin	*	ND	ND	**
Negative control	*	***	ND	ND
Cell without antibody	ND	ND	ND	ND

\*\*\* relatively expressed higher than \*\* and \*

**Figure 1:** Effect of doxorubicin and tiliroside on the percentage of viability of T47D and MCF7 cancer cell lines, (n=3)**Figure 2:** Morphology of alive (a) and dead cell lines (b) T47D (I) and MCF7 (II) (magnification 100 times). [→ : Alive cell; → : Dead cell]

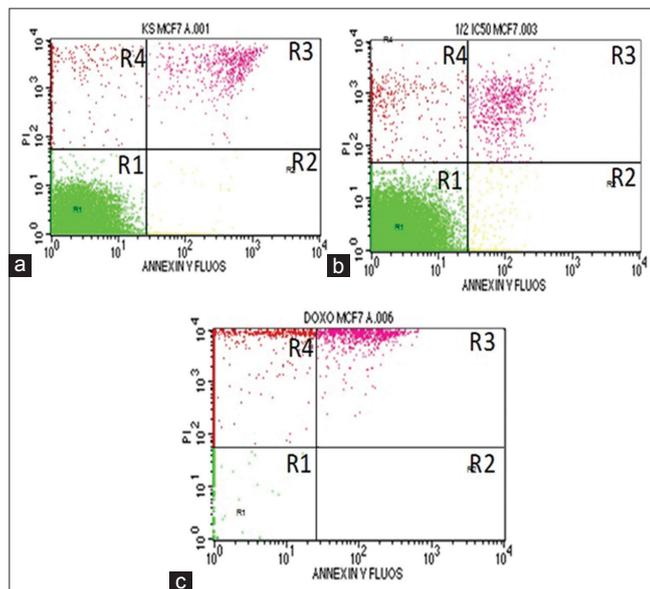
epifluorescence of cells by annexin V or propidium iodide bond which is arrested by UV light.<sup>[25]</sup>

Table 1 shows that the percentage apoptosis of end apoptosis on T47D (4.93%) is greater than MCF7 (3.18%) cell lines. It is indicated that tiliroside showed apoptotic mechanism by the inhibition of cancer cell lines that are more active in T47D compared with MCF7. A striking difference from doxorubicin showed that the mechanism of inhibition on MCF7 was necrosis pathway. It is not surprising because of the characteristic of MCF7 is resistant to doxorubicin. The induction apoptosis process was quite small, even though it is interesting to investigate the effect on molecular levels.

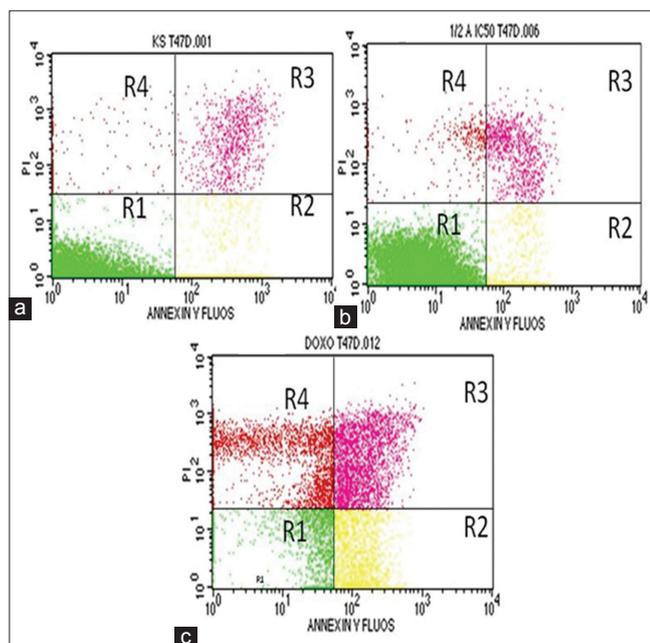
Table 1 shows that tiliroside has minimal activity on apoptosis induction on MCF7 cell lines. It could be caused

by the properties of MCF7, such as wild type cancer cell p53 and overexpression of Bcl-2.<sup>[20]</sup> P53 is a protein which has a role in the repairment of DNA, apoptosis, and cycle cell,<sup>[26]</sup> whereas Bcl-2 is a protein of anti-apoptotic activity.<sup>[27]</sup>

The result showed that tiliroside did not induced apoptosis through intrinsic pathways on T47D because p53 was not expressed (Figure 5). In line with the previous data, Bcl-2 did not express. Similarly with tiliroside, doxorubicin did not induced apoptosis by intrinsic pathways. Consequently, the apoptosis mechanism by extrinsic pathways was mediated by



**Figure 3:** Visualization of apoptosis assay of tiliroside on MCF7. (a) Negative control. (b) tiliroside 50 µg/mL and. (c) doxorubicin 15 µg/mL. The result was representative of duplicate observation



**Figure 4:** Visualization of apoptosis assay of tiliroside on T47D. (a) Negative control. (b) tiliroside 30 µg/mL and (c) doxorubicin 7 µg/mL. The result was representative of duplicate observation.

Caspase 8 and 9. Based on Figure 5, the cytoplasm of T47D colored brown which indicated that tiliroside could activate Caspase 8 and 9.

Table 2 shows the summary of the expression of protein apoptosis of T47D, such as p53, Bcl-2, Caspases 8 and 9. The data show that tiliroside induces apoptosis only through extrinsic pathways (death receptor), marked by an increased expression of Caspases 8 and 9. Expression of p53 and Bcl-2 was not significant.

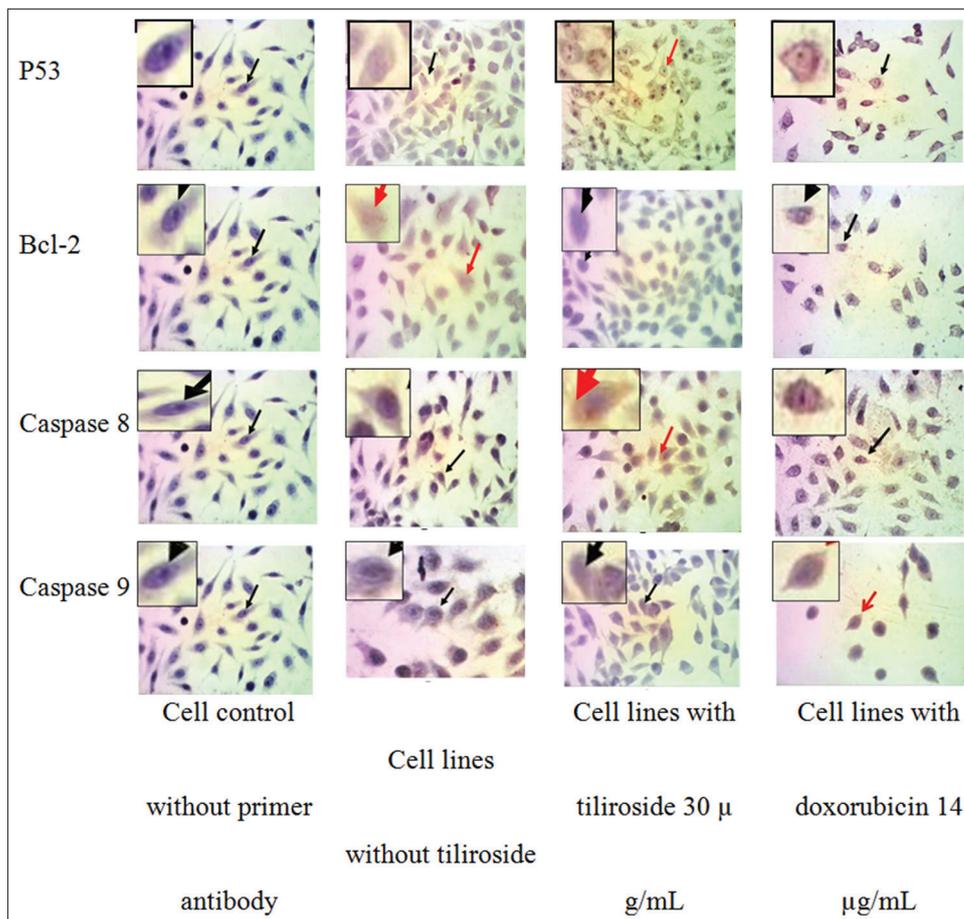
In contrast to T47D cell lines, MCF7 cell lines with tiliroside of 50 µg/ml occurred weak expression of Caspase 8 (Figure 6) moreover Caspase 9 was not expressed. Meanwhile, doxorubicin did not induce the expression of Caspase 9. Even though the expression of Bcl-2 occurred, this expression caused by characteristic of MCF cell lines which overexpression of Bcl-2. Likewise, doxorubicin could not activate Bcl-2. To sum up, the antiproliferative effect of tiliroside was higher in T47 than MCF7.

To strengthen the mechanism of apoptosis, immunocytochemistry was conducted. The principle of this method is based on staining of chromogen DAB which will bind with the antibody of p53, Bcl-2, Caspases 8 and 9, and formed brown color on the cell membrane. Data (Figure 6) showed that MCF7 with tiliroside 50 µg/ml and normal group have weak expression of Bcl-2. In contrast, doxorubicin did not express Bcl-2.

Furthermore, weak brown-colored MCF7 cells were observed by tiliroside 50 µg/ml from the expression of Caspase 8. This indicated that tiliroside activates Caspase 8, as the markers of extrinsic pathways of apoptosis mechanism. On the contrary, doxorubicin did not activate Caspase 8. These results have a similar profile for Caspase 9, in which tiliroside activates Caspase 9 and doxorubicin did not activate it.

## DISCUSSION

From this study, we could know that MCF7 cell line is less responsive to tiliroside and doxorubicin than T47D cell. Welshons et al.<sup>[28]</sup> stated that MCF7 cell line expressed ER<sup>+</sup><sup>[28]</sup> and it is more responsive to chemotherapy than T47D cell, which has the same profile, ER<sup>+</sup>. Other than that, from their study, phenol red contained in media, which is used as pH indicator, is estrogenic. Estrogen stimulation induces the secretion of P-gp that can increase P-gp concentration in MCF7 cell line which has ER $\alpha$ , but not in T47D cell which has ER $\beta$ .<sup>[23]</sup> P-gp in cytoplasm contributes in the progress of chemotherapy, it can pump out chemotherapeutic substances from cells so that the cancers cell can survive.<sup>[29]</sup> In this study, the result was obtained as IC<sub>50</sub> more than 100 µg/ml for MCF7 cell which is different with the study by Tomczyk et al.,<sup>[11]</sup> 21.60±2 µM equivalent with 44 µg/ml. These results from the study by Tomczyk et al.<sup>[11]</sup> showed more potent



**Figure 5:** Visualization immunocytochemistry of expression p53, Bcl-2, caspase 8, and caspase 9 on T47D. Visualization using microscope with magnification 40 times. The result was representative of duplicate observation. [→ : Do not express of protein, → : Expressing protein]

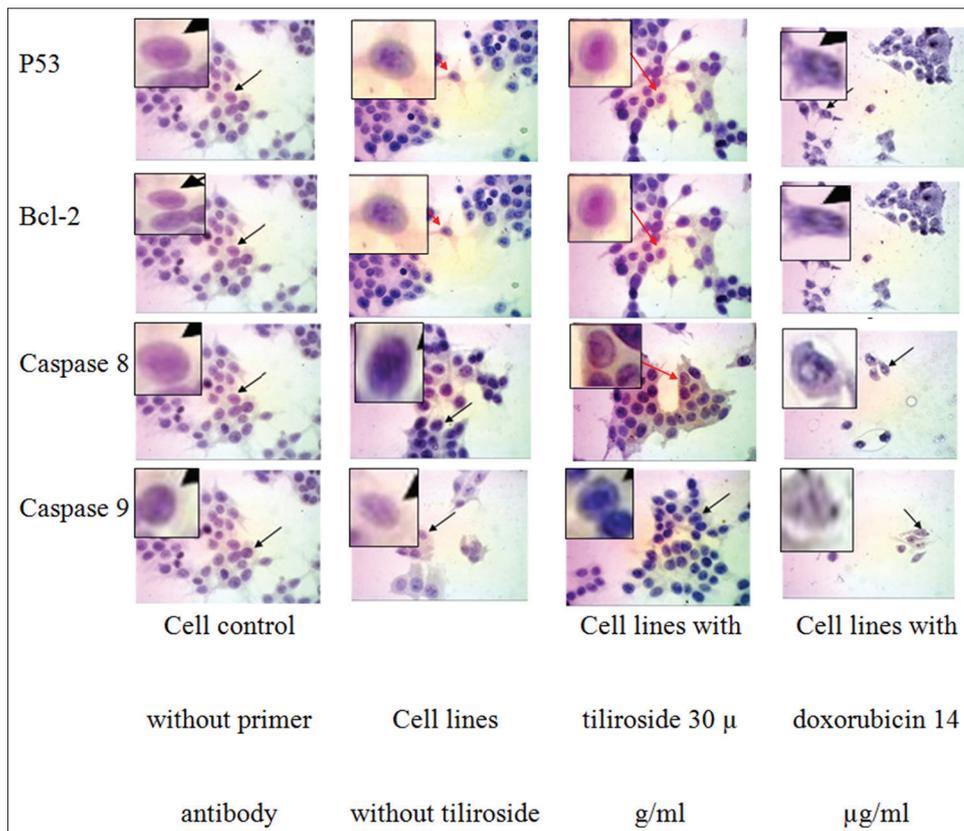
cytotoxic effect of tiliroside on MCF7 cells and no phenol red was used in the media. In general, the activity of tiliroside was more active on T47D than MCF7.

Flow cytometry results showed the induction of tiliroside on apoptosis, so the apoptosis mechanism at molecular levels was conducted by the observed expression of tumor suppressor (p53), protein apoptosis (Bcl-2), Caspases 8 and 9 (apoptosis initiator), using immunocytochemistry method. The basic principle of this method is based on the interaction between antibody (p53, Bcl-2, Caspases 8 and 9) with specific protein in the cancers cell lines. This bonding was detected by applying biotin-labeled secondary antibody. Biotin would bond with horseradish peroxidase enzymes and DAB chromogen and forms brown-colored complex at that bond. Apoptosis is controlled by cycle regulator genes, one of them is p53 gene which is expressed by p53. In general, when DNA damage occurred, p53 would overexpress to repair the DNA damage. Repairment of DNA will be conducted before S-phase through the delay of cycle cell at G1 phase up to the completion of recovery. If the damages of DNA were extensive, consequently, the p53 would activate the apoptosis process.<sup>[30]</sup> In cancer cell lines, p53 frequently have mutation. Furthermore, the cancer cell lines loss the ability

to control cell cycle and to regulate of other proapoptosis proteins.<sup>[31,32]</sup> The characteristic of T47D cell lines is the mutation of gene p53.<sup>[33]</sup> These are nonsense mutations (gene not functionalized) and mis-sense mutations (gene-coded defective protein) at residue 194 (zinc binding domain, L2).<sup>[32]</sup>

Death receptor (Fas) on the surface of cells is one of the TNF (tumor necrosis factor) families. Other families of TNF are NF- $\alpha$  and TNF-related apoptosis including ligand R1 and R2. Apoptosis mechanism through extrinsic pathways will be initiated by the cleavage of death ligand (Fas-L) by cell to interact with death receptors (Fas). Interaction of Fas and Fas-L would form trimerization with adaptor Fas-associated death domain (FAAD). These complexes of death ligand, death receptor, and FAAD are called the death-inducing signaling complex which in the next step activates Caspase 8. Obviously, the activation of Caspase 8 is the initiation of apoptosis. In the following steps, caspase will activate Caspases 6 and 7 resulted apoptosis mechanism through extrinsic pathways.

Expression of Bcl-2 was influenced by the presence of Bcl-2 dimer which causes the cells not to undergo apoptosis through the mitochondrial pathway. It was linear with the result that



**Figure 6:** Visualization immunocytochemistry of expression antibody p53, Bcl-2, caspase 8, and caspase 9 on MCF7. Visualization using microscope with magnification 40 times. The result was representative of duplicate observation. [→ : Do not express of protein, →: Expressing protein]

Caspase 9 is not activated by tiliroside on MCF7. These data strengthen the mechanism of apoptosis of tiliroside through extrinsic pathway as well as Caspase 9 and p53 were not activated by tiliroside. Further investigation on normal cells is needed to get best understanding about the effect of tiliroside on normal and cancer cells.

## CONCLUSION

Tiliroside has higher antiproliferative effect on T47D cancer cell lines compared to MCF7 cancer cell lines. Molecular analysis using immunocytochemistry indicated tiliroside-induced apoptosis through extrinsic pathways by Caspases 8 and 9 activation.

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