Detection of apoptosis mechanism on renal cancer cell treated by 16,17-dehydrodeacetyl-5α-oleandrin compound isolated from *Nerium indicum* Mill. Leaves.

Deteksi mekanisme apoptosis sel kanker ginjal pada pemberian senyawa 16,17-dehidrode-asetil-5α-oleandrin hasil isolasi daun *Nerium indicum* Mill.

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Abstrak

16,17-dehidrodeasetil-5α-oleandrin merupakan senyawa golongan kardenolida yang diisolasi dari fraksi aktif daun *Nerium indicum* Mill (fam. Apocynaceae). Senyawa ini bersifat sitotoksik terhadap berbagai sel kanker dan selektif terhadap sel A498 (kanker ginjal) *in-vitro*, namun mekanisme apoptosisnya belum diketahui. Penelitian ini bertujuan untuk mendeteksi mekanisme apoptosis sel kanker ginjal pada pemberian senyawa 16,17-dehidrodeasetil-5α-oleandrin menggunakan metode fluorescein isothiocyanate-annexin V dan immunositokimia.

Deteksi mekanisme apoptosis terhadap sel A498, dilakukan dengan metode FITC terlabel Annexin V menggunakan Flow Cytometry (FCM). Sedangkan ekspresi protein p53 sel A498 dilakukan dengan metode immunositokimia .

Hasil penelitian menunjukkan bahwa pemberian senyawa 16,17-dehidrodeasetil-5α-oleandrin dosis $3{,}88x10^{-4}$ mM dengan menggunakan metode FITC-annexin V dapat menaikkan persentase jumlah kematian sel antara inkubasi 24 dan 48 jam.

Senyawa 16,17-dehidrodeasetil-5α-oleandrin dosis $1{,}94x10^{-4}$ mM dan $3{,}88x10^{-4}$ mM, dapat menaikkan ekspresi protein p53 secara bermakna ($p<0{,}05$). Persentase ekspresi protein p53 bertambah seiring dengan waktu inkubasi sampel.

Kata Kunci: 16,17-dehidrodeasetil-5α-oleandrin,fluorescein isothiocyanate-annexin V, imunositokimia, sel A498

Abstract

The 16,17-dehydrodeacetyl-5α-oleandrin was isolated from an active fraction of *Nerium indicum* Mill leaves (fam. Apocynaceae). This compound was cytotoxic against various cancer cells, and selective on A498 cells (Renal cancer). However, the apoptosis mechanism was still unknown yet. Therefore, the aim of this study was to know the apoptotic mechanism of 16,17-dehydrodeacetyl-5α-oleandrin on A498 cells by FITC labeled annexin V and immunucytochemical assays.

The detection of apoptotic mechanism on A498 cells was performed with FITC-conjugated annexin V using Flow Cytometry. The p53 protein expression were detected using immunocytochemical.

Treatment with 16,17-dehydrodeacetyl-5α-oleandrin (3.88 x 10^{-4} mM) using FITC-annexin V increased the percentage of the dead cells in the 24th and 48th hours incubation period. The 16,17-dehydrodeacetyl-5α-oleandrin (1,94x10^{-4} and 3,88x10^{-4} mM) raised significanly p53 protein expression ($p<0,05$). The percentage of the p53 protein expression increased throughout the time of samples incubation.

Key words: 16,17-dehydrodeacetyl-5α-oleandrin,fluorescein isothiocyanate-annexin V, immunocytochemically, A498 cells.

Introduction

Nerium indicum Mill. has been reported to be an effective analgetic, central nervous system depressant, and an antileukaemia (Zia *et al*., 1995) while *Nerium oleander* extract (Anvirzel) induces the dead cancer cells (Pathak *et al*., 2000). Another study on cardiac glycosides showed that the Ca^{+2} was stimulated and the apoptosis on prostat cancer was raised due to treatment of the cardiac glycosides (Mc Conkey *et al*., 2000).

Phytochemical studies showed that *Nerium* sp. contain neriodorin, carabin, tanat acid, kanerin, 12,13-dehidroursulat acid, oleandrin, kanerat acid, nerikumarat acid, isonerikumarat acid, oleanderol, oleanderen, kanerodione, kanerin, neriumin, neriuminin and neridiginoside (Siddiqui *et al*., 1989; Begum *et al*., 1999). β-Oleandrin was the major compound present in the leaves of *Nerium indicum*, and the principal bioactive compound isolated by bioassay BST (*Brine Shrimp Lethality Test*) guided isolation method. The LC_{50} of β-oleandrin was 2,36 x 10^{-6} M on BST (Wahyuningsih *et al*., 2000a). Furthermore, β-oleandrin inhibited the growth of myeloma cells at 1,74 x 10^{-5} M that was comparable to 3,40 x10^{-5} M of vincristine sulphate (Wahyuningsih *et al*., 2000b). In addition, β-oleandrin was cytotoxic on several cancer cells *in vitro*, unfortunately this compound was also cytotoxic to normal cells as well (Wahyuningsih, 2006).

The 5-α configuration oleandrin was also isolated from the leaves of *N. Indicum*, identified as 16,17, dehydrodeacetyl-5α-oleandrin (Figure 1). This compound was selectively cytotoxic on kidney cancer cells (A498, IC_{50}, 14,34 x 10^{-6} mM), and non toxic against normal cells that was different from the 5-β conformation (Wahyuningsih, 2008). A study on the apoptotic mechanism of A498 cells upon treatment of 16,17, dehydrodeacetyl-5α-oleandrin has not been reported, so we are reporting our study on this matter using fluorescein isothiocyanate labeled annexin V and immunocytochemical assays.

Methodology

Materials

16,17-dehydrodeacetyl-5α-oleandrin (isolated from the leaves of *N.indicum* Mill.) (Figure 1), the A498 cells was obtained from Erasmus Medical Center, Netherland, Fetal bovine serum (FBS) (Gibco BRL), medium Roswell Park Memorial Institute (RPMI) 1640 (Sigma), *Phosphate Buffered Saline* (PBS), streptomisin, penisilin, glutamin, DMSO (E. Merck), tris(hydroxymethyl) amino-methane (Tris base), alkohol absolut, asam trikhlorasetat (TCA), Kloroform, Methanol (MeOH), FITC-Annexine V (BioWhittaker, Verviers, Belgium), antibody p53 [(DAKO LSAB 2 KIT) Dako Corporation].

Stock Solution Preparation

The 16,17-dehydrodeacetyl-5α-oleandrin (1.0 mg) was dissolved in 100,0 μL of dimethylsulfoxide (DMSO) (E Merck) and then RPMI-1640 medium was added to obtain 10 mg/mL of stock solution.

In Vitro A498 Cells Culture

The renal cancer (A498) cells was maintained *in vitro* in RPMI-1640 medium (Sigma Chemical Co., USA), supplemented with 10 % fetal bovine serum (Gibco Invitrogen,USA), 100 μg/mL of strepto-mycine (Gibco Invitrogen,USA), 100 unit/mL of penicillin (Gibco Invitrogen, USA), and 2 mM of glutamin in tissue culture flask. The cells were incubated in 5 % of CO_2 incubator set at 37 ^oC.

Fluorescein Isothiocyanate-V Test

The A498 cell treated with 16,17-dehydrodeacetyl-5 α-oleandrin were incubated in RPMI-1640 medium at 37^oC for 24, 48 and 72 hours, and then were removed from the flask adding tripsin to them. The cells suspension was moved into a centrifuge tube and centrifugated at 1500 RPM for 5 minute. The palette was removed by pouring supernatan to another tube, and then 1 mL of FITC (*Fluorescein Isothiocyanate*) labeled annexin V

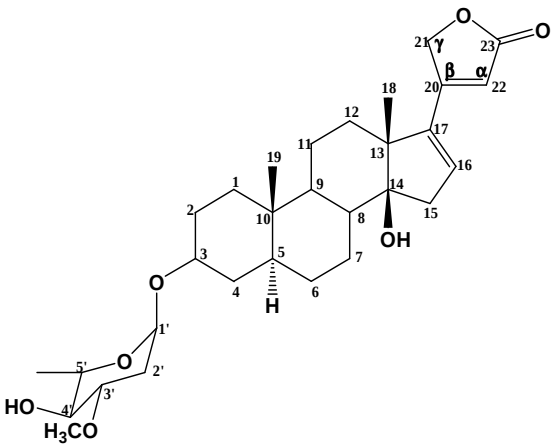

Figure 1. Structure of 16,17-dehydrodeacetyl-5α-oleandrin.

was added, and incubated at 37°C for 1 hour. The next step, the palette was to centrifuse for 5 minute at a speed of 1500 RPM. The supernatan was thrown away and only the palette remained to be washed with a medium without fenol, and then analyzed using *Flow Cytometry* (FCM). The percentage of the cell apoptosis was calculated by the following formula:

$$\% \text{ apoptosis} = \frac{\text{Number of dead cells (M1)}}{\text{Number of cells in view (All)}} \times 100\%$$

Immunocytochemical Test

The A498 cell was cultured in a 24-well plate with each well containing 1.10^5 cells in 500 µL of RPMI-1640 medium, supplemented with 0,5 % FBS, then the cells were incubated in 5 % of CO_2 incubator at 37 °C for 12 hours. The next step was removing the medium, followed by adding ($1{,}94 \times 10^{-4}$ mM and $3{,}88 \times 10^{-4}$ mM) in 500,0 µL RPMI 1640 with 10 % FBS. The compound was tested (n=3) on a 24-well culture plate. The plate containing cells and samples was incubated at 37 °C in 5 % of CO_2 incubator for 24, 48 and 72 hours. After certain periods (24, 48, and 72 hours) of times, the cells were added with tripsine, released from the flask. The suspension cell was centrifuged at 1500 RPM for 5 minutes. The cells deposit was resuspended with a medium, and then made into a smear on a poly-lysine slide. The smear was stucked with acetone (E. Merck) to the poly-lysine slide and let it stands for 10 minutes, and then washed with PBS twice. The object glass was entered into H_2O_2, let it there for 20 minutes, and then was washed in PBS for five minutes. Finaly, the serum blocking was performed for 15 to 20 minutes. A primary antibody was added to it and left to stay for 1 hour in a damp box or in a refrigerator for a whole night, and then washed in PBS for 3 up to 5 minutes. Drops of biotinylated secondary antibody solution was added to the object glass and was let stay for 30 minutes, and then washed in PBS for 3 up to 5 minutes. The next process was the incubation of the object glass in streptavidin (Novostain) for 30 minutes, followed by washing it in PBS two times for 5 minutes. The glass was then incubated in cromogen 3,3-diaminobenzidin/DAB (Lab Vision) for 3 to 8 minutes, washed in PBS, and then rewashed in aquadest. The next step was to stain the object with hematoxilin (Dako) for 3 to 4 minutes and wash it in aquadest, fixed with mounting and covered with deg glass. The p53 protein expression was examined under a UV light microscope.

Analysis:

$$\% \text{ p53 Expression} = \frac{\text{Number of cells expressing p53 protein}}{\text{100 cells}} \text{ X } 100\%$$

Result And Discussion

Determination of the apoptosis mechanism of 16,17-dehydrodeacetyl-5α-oleandrin was innitiated with qualitative and quantitative observations of the apoptosis performance of the kidney cancer cell culture (A498). Quantitatively the performance of the apoptosis of the kidney cancer cell (A498) was detected by Flow cytometry using FITC method labeled Annexin V (Koopman *et al.,*1994; Boersma *et al.,* 1996). This method could also be used to detect early apoptosis as well as to calculate the dead cells (Zhang *et al.*,1997).The result of the apoptosis quantitative calculation using Flow cytometry was presented in Figure 2. The apoptosis calculation of the kidney cells (A498) apoptosis used FITC (*Fluorescein isothio cyanate*) labeled Annexin V which indicated that 16,17-dehydrodeacetyl-5α-oleandrin ($1{,}94 \times 10^{-4}$ mM) increased the percentage of the dead cells on 24 and 48 hours incubation period (Figure 2).

Incubation for 48 to 72 hours did not give any significance deferences, because 94,80 % of the cells had died in the 48 hours of incubation. The same result was also obtained when it was treated with 16,17-dehydro-

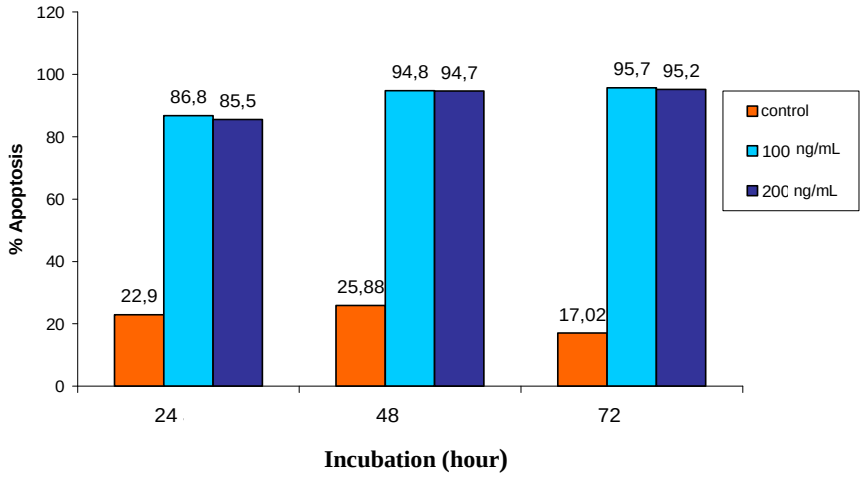

Figure 2. Percentage of A498 cell apoptosis after treatment with 1,94 x10^{-4} mM and 3,88x10^{-4} mM, doses of 16,17-dehydrodeacetyl-5α-oleandrin, incubated for 24, 48 and 72 hours

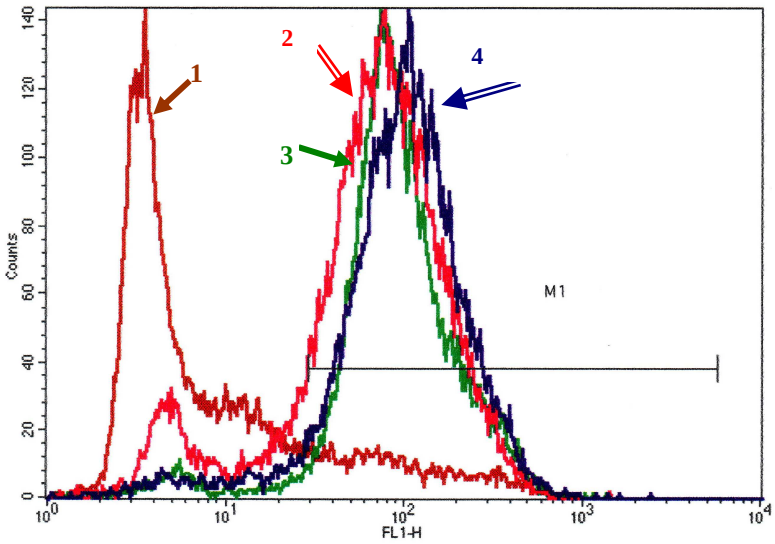

Figure 3. Histogram of A498 cells undergoing apoptosis due to 16,17-dehydrodeacetyl-5α-oleandrin (3,88x10^{-4} mM) incubated for 24, 48 dan 72 jam.

Notes: 1) Control A498 cells (48 jam)
2) 16,17-dehydrodeacetyl-5α-oleandrin, incubated for 24 jam
3) 16,17-dehydrodeacetyl-5α-oleandrin, incubated for 48 jam
4) 16,17-dehydrodeacetyl-5α-oleandrin, incubated for 72 jam

deacetyl-5α-oleandrin at 3,88x10^{-4} and 1,94x10^{-4} mM. Based on the above data, it was concluded that treatment with 16,17-dehydrodeacetyl-5α-oleandrin at 1,94 x10^{-4} and 3,88x10^{-4} mM doses did not affect the number of the dead cells. The incubation period that affecting the cells'death was the incubation times at of 24 and 48 hours period. This led to a temporary conclution that 16,17-dehydro-deacetyl-5α-oleandrin caused an early death on

the A498 cells in the the 48-hour period. The percentage of the dead cells was determined on the number of the M1 dead cell divided by the number of cells in fiew (All) times 100 %. These M1 cells which represented the number of cells undergoing apoptosis as plasma membrane could bind FITC-Annexin V while the plasma membrane of normal cells could not bond FITC-Annexin V (Zhang *et al.*, 1997). The number of cells undergoing apoptosis increased alongside the incubation period of sample cells and reached the optimum during the 48-hour incubation period.

This result was parallel to the data reported by Boersma (1996). This apoptosis response using FITC-labeled Annexin V assay increased alongside the incubation period of cisplatin in the cell cultur of Chinese Hamster Ovary. A similar study was reported by Zhang (1997) that FITC-labeled Annexin V could be used to detect the plasma membrane of the cells undergoing early apoptosis as marked by dark and light fluorescence. After the apoptosis process had ended, only light fluoresceins was seen. The dark flourescens showed the cells undergoing early apoptosis, marked with the presence of intact cell membrane, containing normal DNA, only a few damage DNA and chromatin condenzations. The flourescens light showed the population of cells which had their cell nuclei fragmented,and the membrane blebbed in the end of the apoptosis process (Zhang *et al.,* 1997). The increasing in apoptosis along with the incubation period of the compound was also observable in the histogram (Figure 3). It was clear that at the 48

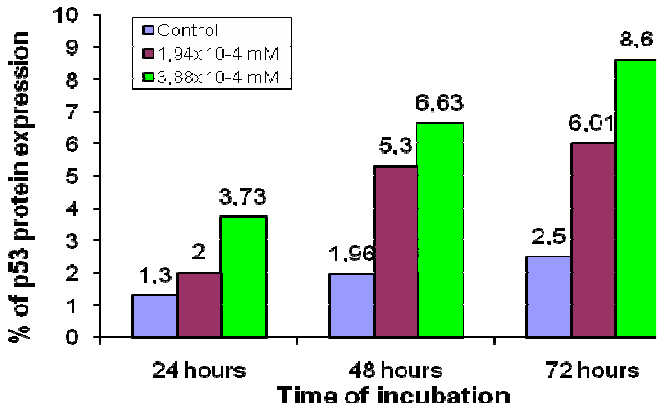

Figure 4. Percentage of p53 protein expression of renal cancer cells (A498) after treatment with 16,17-dehydrodeacetyl-5α-oleandrin (1,94 and 3,88 x 10^{-4} mM), incubated for 24, 48 and 72 hours.

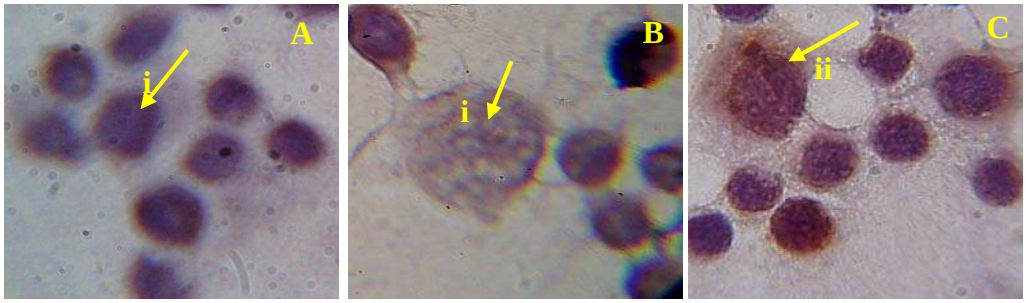

Figure 5. Immunocytochemical of p53 protein expression of the A498 cells, incubated for 48 hour (A) Control, (B) 16,17-dehydrodeacetyl-5α-oleandrin, 1,94x10^{-4}mM doses (C) 16,17-dehydrodeacetyl-5α-oleandrin, 3,88x10^{-4} mM doses (200x)

Note: i. Cells not expressing p53 protein.
ii. Cells expressing p53 protein.

hour incubation of the cell control did not show apoptosis, whereas the cell treated with 3,88 x 10^{-4} mM of the compound for 24, 48 and 72 hour incubations indicated an increase in the number of the dead cells.

Using immunocytochemical assay (Carson, 2000), the apoptotic mechanism approach of 16,17-dehydrodeacetyl-5α-oleandrin was carried out by way of observing the p53 protein expression of Renal cancer cells (A498). The p53 protein is a tumor supressor protein and has 53 kilodalton of Molecular weight activated by DNA damage or certain stress of cells. This protein can induce apoptosis with the increase of Bax expression. This gene disguises as a Bax protein which plays the role in the apoptosis process (Meiyanto, 2002).

Immunocytochemical assay provides qualitative and semi-quantitative data. The cells is observable microscopically qualitatively, and the p53 protein expression can be quantate by counting the cells. The p53 protein expression cells are marked with blackish brown nuclear cells. On the other hand, cells which are not p53 protein expression are marked with blue nucley. The cells damage because of the 16,17-dehydrodeacetyl-5α-oleandrin treatment, can be seen through a microscope (Figure 4), while the the percentage of p53 protein expression can be calculated deviding the number of the p53 protein expression cells by 100 cell and multiply by 100 % (Figure 4).

There is significant different (one-way Anava $p<0,05$), between the control group and the groups treated with 16,17-dehydrodeacetyl-5α-oleandrin (1,94x10^{-4} and 3,88x10^{-4} mM) upon 24-, 48-, and 72- hours incubations. Treatment with 16,17-dehydrodeacetyl-5α-oleandrin (1,94x10^{-4} and 3,88x10^{-4} mM) significantly increases p53 protein expression ($p<0,05$). Treatment after 48 hour, the increase in percentage of p53 protein expression was not as large as the one aftar 24 hour incubation (both 1,94x10^{-4} mM and 3,88x10^{-4} mM). The half life of p53 protein is short, but there are several p53 proteins that have undergone mutation. This was due to the anti p53 antibody used to detect p53 which undergone or not yet undergone mutation. The result was confirmed by the number of the dead cells that counted by FITC-Annexin V method (*flow cytometry*) (Boersma *et al.,* 1996).

The immunocytochemical test (Figure 5) showed that treatment with 16,17-dehydrodeacetyl-5α-oleandrin for 48-hour, the A498 cells expresssed p53 protein more than that of the control group [1,94x10^{-4} mM (Figure 5.B); and 3,88x10^{-4} mM (Figure 5.C)] upon 48 hours incubation. The form of the cells was different from those of the control group (Figure 5.A).

The cells group treated with 16,17-dehydrodeacetyl-5α-oleandrin expressed p53 protein with the condensation of their nuclei and shrank indicating apoptosis occured. These apoptotic symptoms were seen on the cells received treatment with 16,17-dehydrodeacetyl-5α-oleandrin at both doses 1,94x10^{-4} mM and of 3,88x10^{-4} mM. The 16,17-dehydrodeacetyl-5α-oleandrin could increased p53 protein expression which was up-regulation of caspase activity through Bax protein. Together with other protein, the Bax protein will activate *cytochrom c* released from mitochondria. What follows is a chain of activities on *caspase 9* and then on *caspase 3,* resulting in apoptosis (Meiyanto, 2002).

Conclusion

1. The percentage of the dead cells increased during 24th and 48th hours incubation after treatment with 16,17-dehydrodeacetyl-5α-oleandrin (3,88 x10^{-4} mM).
2. The 16,17-dehydrodeacetyl-5α-oleandrin (1,94x10^{-4} and 3,88x10^{-4} mM) raised significanly p53 protein expression ($p<0,05$). The percentage of the p53 protein expression increased throughout the time of the sample incubation.

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