

## Pinostrobin isolated from *Kaempferia pandurata* Roxb induced apoptosis in T47D human breast cancer cell line

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### Abstract

Pinostrobin isolated from *Kaempferia pandurata* Roxb. which belongs to the family of Zingiberaceae, was observed to induce apoptosis in T47D human breast cancer cell line. Pinostrobin at 10, 50, 100 µg/ml for 24, 48 and 72 h induced DNA fragmentation and increased the percentage of apoptotic cell after acridine orange – ethidium bromide staining. Our result demonstrated that pinostrobin can induce apoptosis in in T47D human breast cancer cell line in a time and concentration dependent manner by increase expression of p53, bax, caspase-3 and decreased expression of bcl-2 by immunohistochemical analysis.

**Keywords :** Pinostrobin, *Kaempferia pandurata* Roxb., T47D cell, Apoptosis, p53

### INTRODUCTION

*Kaempferia pandurata* Roxb. is a traditional medicinal herb which belongs to the family of Zingiberaceae, grown the moist, shady areas of India, China, Indonesia and throughout Southeast Asia and has been used for antitussive, antidiarrhoeal, diuretic, anti-inflammatory. According to several phytochemical reports, major constituents of the rhizoma of *Kaempferia pandurata* Roxb. are composed flavonoids as pinostrobin and reported that have anticancer activity in vitro in many other tumor cell lines, myeloma (Sukardiman *et al.*, 2000) and human breast cancer (Bail *et al.*, 2000). Sukardiman *et al.* (2000) recently found the activity inhibitory effect of DNA Topoisomerase I inhibitor so these result suggest that inhibitory effect from pinostrobin contribute to its cytotoxicity by DNA fragmentation and inducing apoptosis. In the present study was performed to determined the apoptosis-inducing effect of pinostrobin isolate from *Kaempferia pandurata* Roxb. in T47-D human breast cancer cell line .

### MATERIAL AND METHOD

**Plant material.** Rhizome of *Kaempferia pandurata* Roxb. were collected in September 2005 from Mojokerto, East Java, Indonesia. Identification was made by Mr I.G.P Santa and a voucher specimen No KP 102 was deposited at the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia.

**Isolation of pinostrobin.** The rhizome of *Kaempferia pandurata* Roxb (300g) was extracted with n-hexane using maceration method and concentrated *in vacuo* to yield n-hexane extract (32g). The n-hexane extract was subjected to silica column chromatography by gradient development of chloroform : methanol solvent system. The pinostrobin crystal was recrystallized by hot methanol. Spectral data of IR, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR and MS of isolate was in accordance with those of pinostrobin as already reported in the literature .

**Cell Culture.** T47-D human breast cancer cell line were obtained from NAIST, Narita, Japan. Cell were grown in RPMI-1640 media (Sigma,US) containing 10% FBS, 100 mg/L Streptomycin and 10<sup>5</sup> unit Penicillin. A humidified incubator with 5% CO<sub>2</sub> was used to row the cell at 37°C.

**Chemicals.** The p53, bax and bcl-2 antibody was from Boeringer Mannheim, Germany. All other chemicals were purchased from Sigma, St.Louis,MO.

**Cell morphology.** Cell morphology was determined by examining the culture cell under inverse microscope at magnification of 100x. Untreated cell served as control.

**Determination of Cell Viability.** Cell were plated in triplicate at a density of 1 x 10<sup>5</sup> cell/ml in dish. Cell were treated with 10, 50, 100 µg/ml pinostrobin for 24, 48 and 72 hours incubation. At the end of treatment, cell were harvested and counting using a haematocytometer. Cell viability was assessed by trypan blue exclusion test. Percentage of cell viability was calculated from the formula:

$$\text{Percentage of cell viability} = \frac{\text{total cell} - \text{cell death}}{\text{total cell}} \times 100\%$$

**Determination of Apoptotic Cells.** Cell were treated with 10, 50, 100 µg/ml pinostrobin for 24, 48 and 72 hours incubation. Apoptosis was determined by morphological analysis after Acridine Orange / Ethidium Bromide (AO/EB) staining, a previously described . A minimum of 300 cell was examined for each case and the result expressed as number of apoptotic cells over total number of cell counted. Percentage of apoptotic cell was calculated from the formula :

$$\text{Percentage of apoptotic cell} = \frac{\text{Amount of apoptotic cell}}{\text{Total cell examined}} \times 100\%$$

The data viability cell and apoptotic cell were expressed as mean ± SEM of triplicate. Result were analysed statistically by One-way ANOVA followed by Tukey's multiple comparison using SPSS software student's version 14.0. The difference was considered significant if p<0.05.

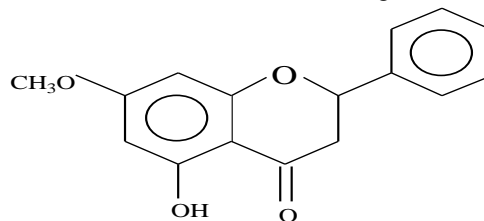
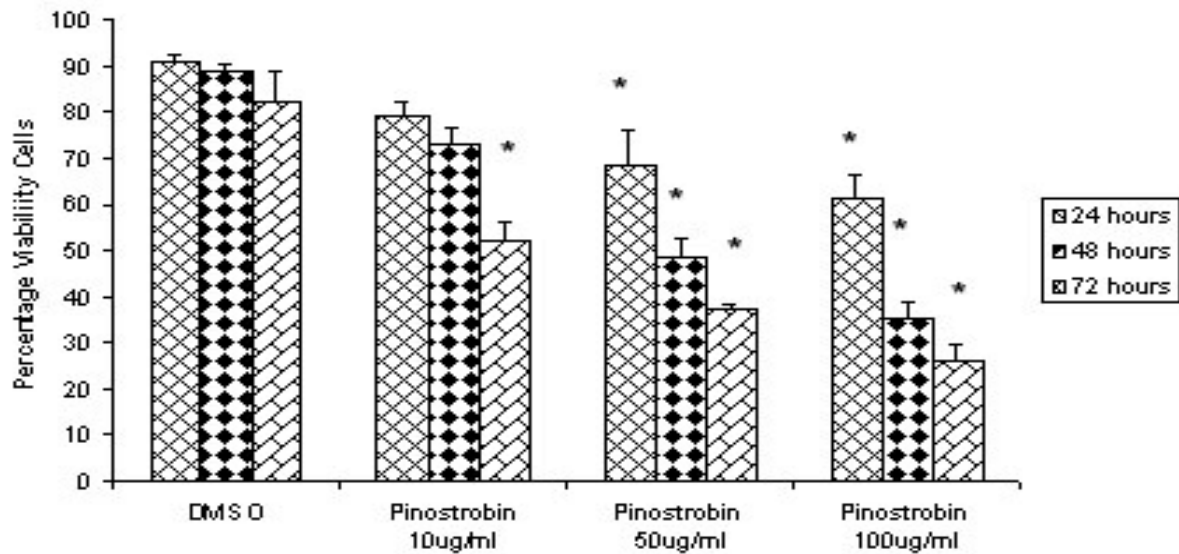
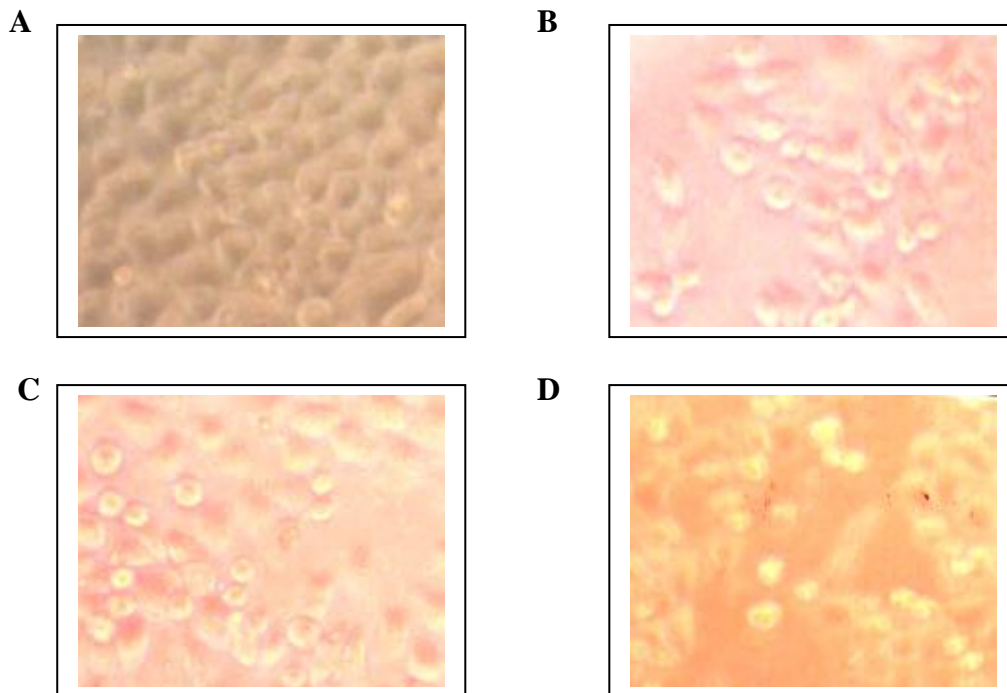


Fig 1. Structural formula of pinostrobin

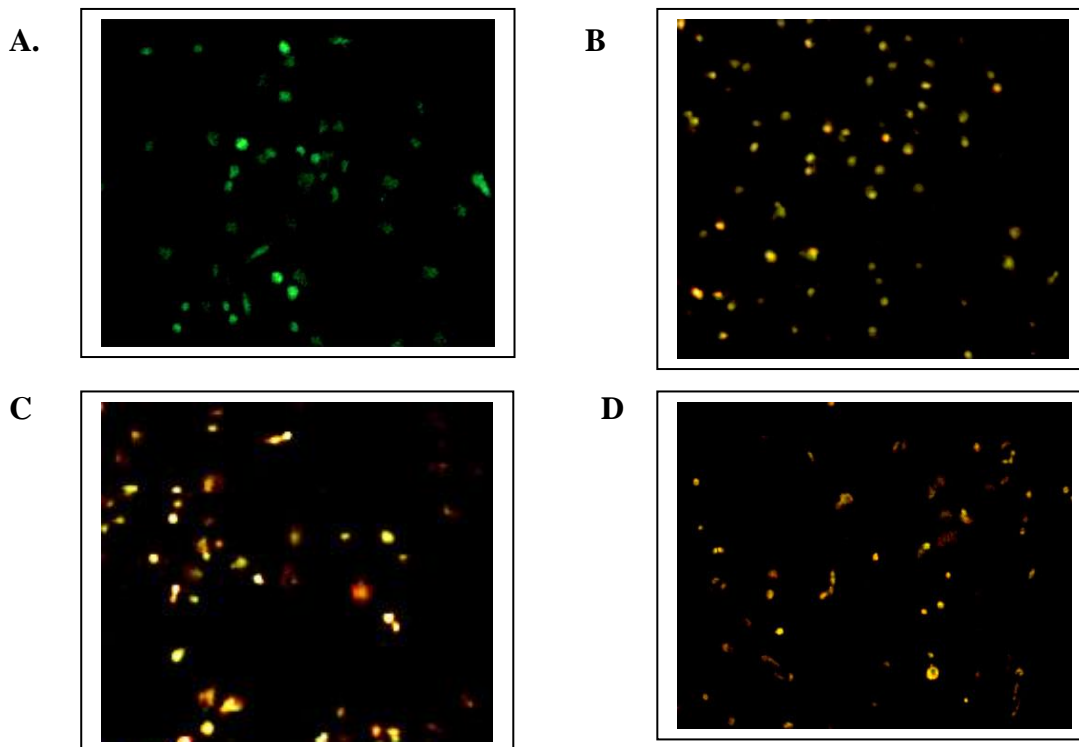
## RESULT AND DISCUSSION



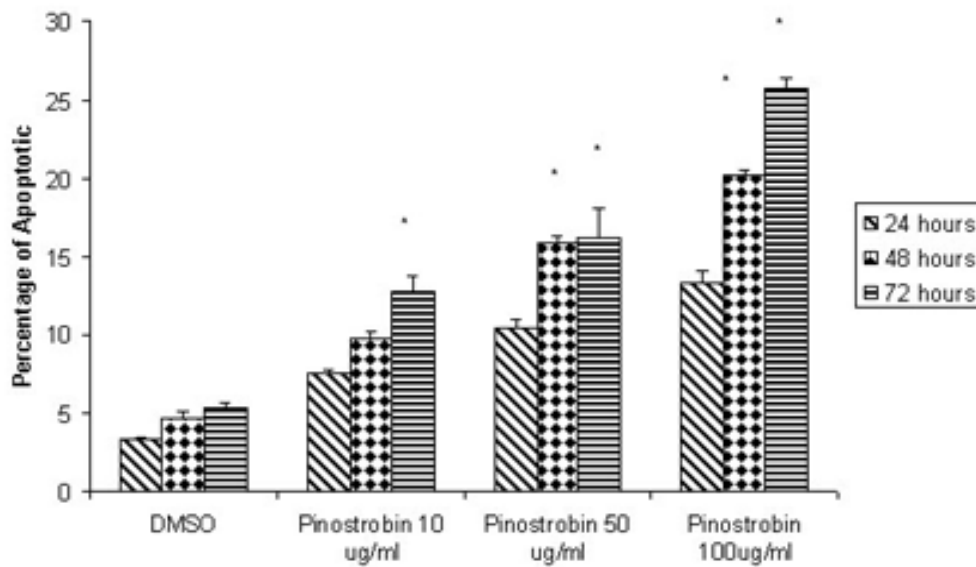
**Fig. 2.** Effect of pinostrobin on cell viability of TD-47 human breast cancer cells. Cell were treated with control DMSO; at 10, 50, 100  $\mu\text{g/ml}$  pinostrobin 24, 48 and 72 h, and cell viability was determined by trypan blue exclusion. Data represent mean  $\pm$  SEM of triplicate. \* $p < 0.05$  compared to control (One Way ANOVA followed by Tukey's multiple comparison test).



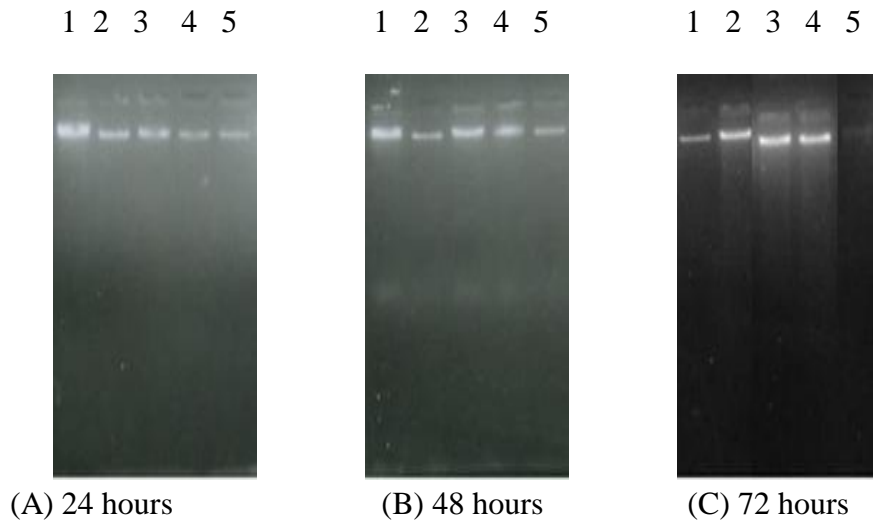
**Fig. 3.** Effect of pinostrobin on morphology change of T47D human breast cancer cells (phase-contrast microscopy). A. untreated cells cultured (DMSO 0.1%). B. cells were treated with 10  $\mu\text{g/ml}$  pinostrobin. C. Cells were treated with 50  $\mu\text{g/ml}$  pinostrobin D. Cells were treated with 100  $\mu\text{g/ml}$  pinostrobin for 48 h.



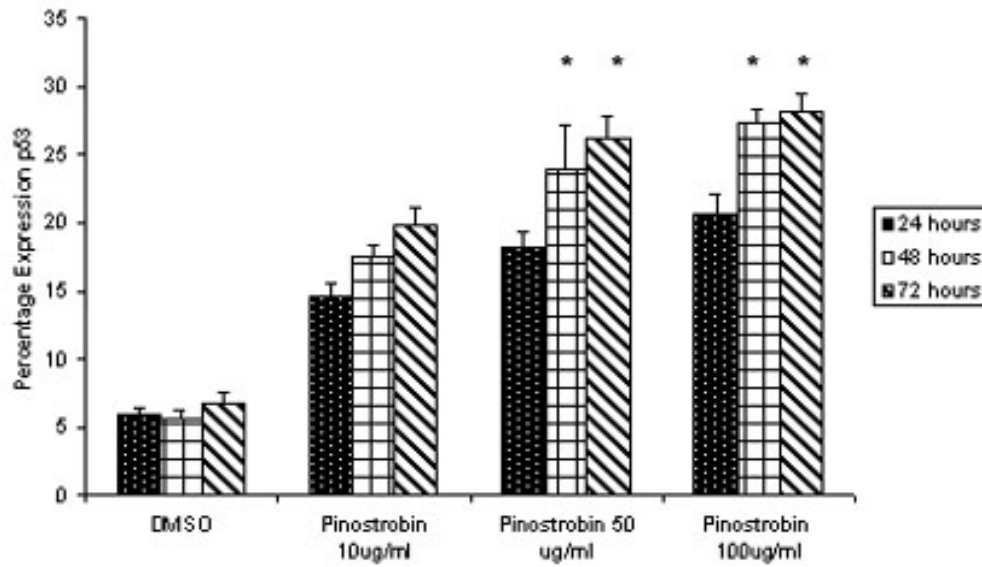
**Fig. 4.** Effect of pinostrobin on induction apoptosis of T47-D human breast cancer cells , analysis by Acridine Orange/Ethidium Bromide (AO/EB) staining (examined using flourecent microscopy). A. untreated cells cultured (DMSO 0.1% ). B. Cells were treated with 10 µg/ml. C. Cells were treated with 50 µg/ml D. Cells were treated with 100 µg/ml pinostrobin for 24 h.



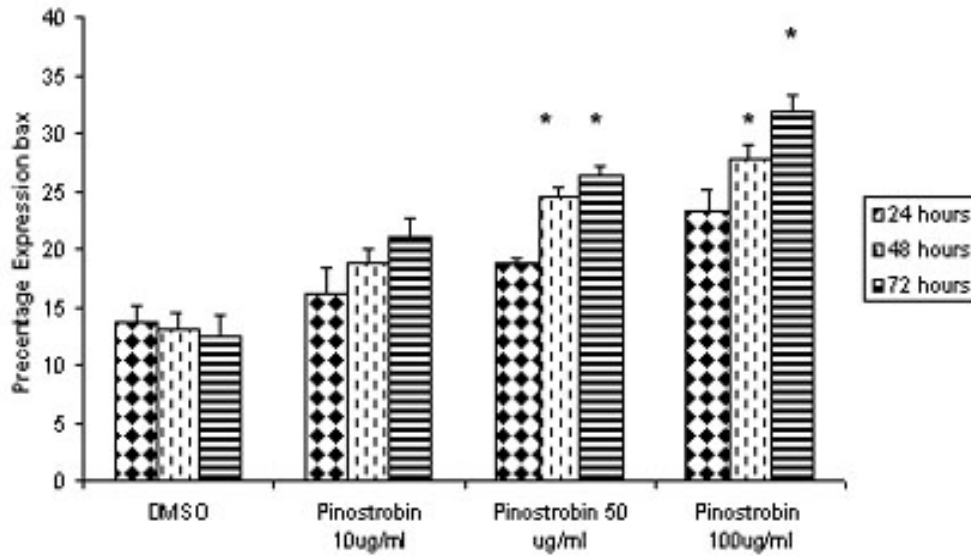
**Figure 5.** Relative apoptosis induced by pinostrobin as determined by Acridine Orange - Ethidium Bromide (AO / EB) staining. The percentage apoptotic cell was examined for each case and the result expressed as number of apoptotic cells over total number of cell counted . Data represent mean  $\pm$  SEM of triplicate. \*  $p < 0.05$  compared to control (One Way ANOVA followed by Tukey's multiple comparison test).



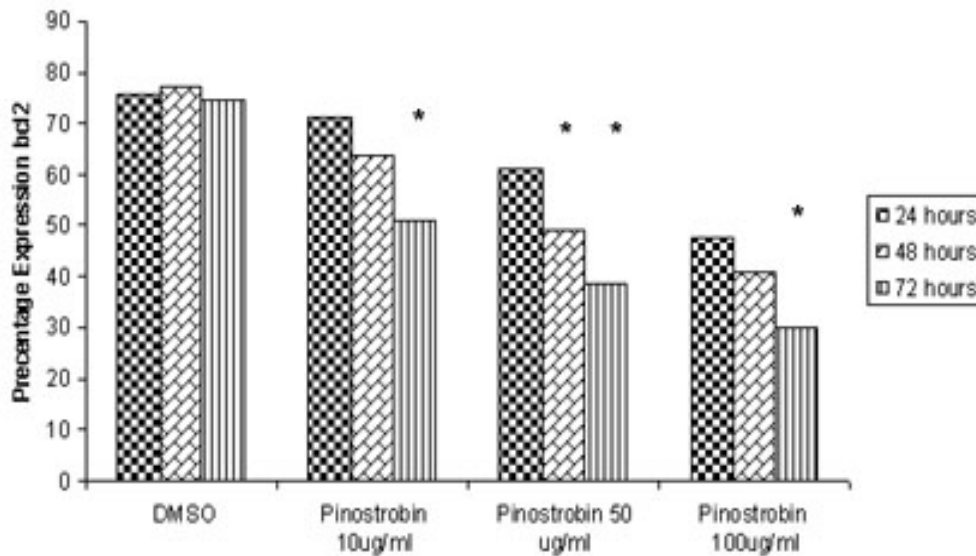
**Fig. 6.** Concentration course experiment for pinostrobin - induction internucleosomal DNA fragmentation T47D human breast cancer cells, analysis by electrophoresis gel. Lane1 untreated cells cultured (DMSO 0,1%). Lane 2 cells were treated with 100 µg/ml pinostrobin. Lane 3 cells were treated with 100 µg/ml pinostrobin Lane 4 cells were treated with 100 µg/ml pinostrobin.



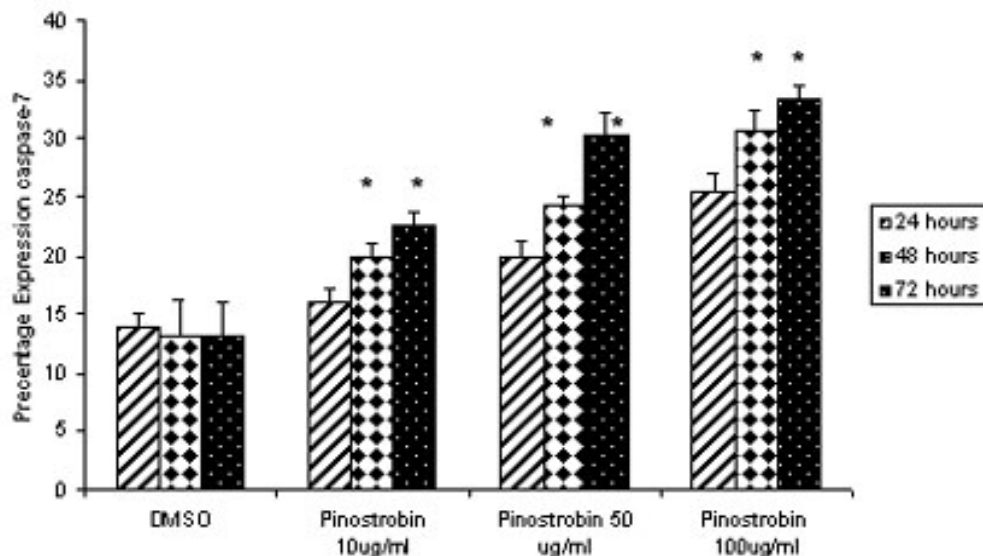
**Figure 7.** Concentration course experiment for pinostrobin-induction increased expression of p53 from T47D human breast cancer cells , analysis by immunohistochemical.. Data represent mean ± SEM of triplicate. \*p < 0.05 compared to control (One Way ANOVA followed by Tukey’s multiple comparison test).



**Figure 8.** Concentration course experiment for pinostrobin - induction increased expression of bax from T47D human breast cancer cells, analysis by immunohistochemical. Data represent mean ± SEM of triplicate. \*p < 0.05 compared to control (One Way ANOVA followed by Tukey’s multiple comparison test).



**Figure 9.** Concentration course experiment for pinostrobin - induction decreased expression of bcl-2 from T47D human breast cancer cells, analysis by immunohistochemical. Data represent mean ± SEM of triplicate. \*p < 0.05 compared to control (One Way ANOVA followed by Tukey’s multiple comparison test).



**Figure 10.** Concentration course experiment for pinostrobin - induction increased expression of caspase-7 from T47D human breast cancer cells, analysis by immunohistochemical. Data represent mean  $\pm$  SEM of triplicate. \*  $p < 0.05$  compared to control (One Way ANOVA followed by Tukey's multiple comparison test).

The results showed the morphological changes of T47D breast cancer cells which leads to a characteristic of apoptosis in cells that are added pinostrobin. Cancer cells become shrunken and chromatin condensation was observed. The results also showed that the higher the concentrations and the addition of the incubation time, the more cell death by apoptosis.

Qualitatively apoptosis can be detected by looking at the future of the DNA fragmentation using gel electrophoresis as done by Chopin (2002). DNA fragmentation can indicate cells undergoing apoptosis. Qualitative test results can be seen in Figure 6. On analysis by agarose gel that can inform both qualitatively and quantitatively that pinostrobin of *Kaempferia pandurata* at a concentration of 10; 50 and 100 $\mu$ g/ml good at incubation time 24, 48 and 72 hours seems polish band indicates the presence of increasing concentrations apoptosis. dengan pinostrobin incubation time and the addition of more and more evident DNA fragmentation, especially at 100 $\mu$ g/ml concentration with an incubation time of 72 hours seems polish DNA is more clear than T47D breast cancer cells.

Ability pinostrobin compounds to cause DNA fragmentation in T47D breast cancer cells were related to its activity as an inhibitor of DNA topoisomerase (Sukardiman et al., 2003). DNA topoisomerases are enzymes that have important functions in the intracellular processes of cancer cells, among others, play a role in the process replicas, transcription, DNA recombination and cancer proliferasisel process (Pommier, 1993; Hsiang, 1995). By inhibiting the activity of DNA topoisomerase by inhibitor compounds, the process of tertiary bonding between the enzyme with the longer DNA of cancer cells. So it will form a protein linked DNA breaks (PLDB), resulting in cancer cell DNA damage and subsequent

cessation of cell replication and ends with cell death (Joseph, 1989; Hsiang, 1995).

At the time of T47D breast cancer cell incubation for 24, 48 and 72 hours when compared with the control group with the addition pinostrobin 10; 50 and 100 $\mu$ g/ml showed an increase number of apoptotic cells, increased expression of p53, bax, caspase-7 and a decrease in the expression of bcl-2 and COX-2 significantly.

**Conclusion.** Pinostrobin dari temu kunci (*Kaempferia pandurata* Roxb) memiliki aktivitas induksi apoptosis terhadap sel kanker payudara T47D sevara in vitro melalui mekanisme aktivasi p-53- bax dan hambatan ekspresi bcl-2 dan COX-2.

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