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TAHUN ANGGARAN 2009

JUDUL

PINOSTROBIN ISOLATE FROM *KAEMPFERIA PANDURATA* ROXB INDUCED APOPTOSIS IN TD-47 HUMAN BREAST CANCER CELL LINE

Ketua Peneliti :
Prof.Dr.Sukardiman,Apt.MS

Anggota :
Prof.Dr.Noor Cholies Zaini,Apt
Dr.Aty Widyawaruyanti,Apt
Prof.Dr.Sismindari,Apt
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Dibiayai oleh Dikti, Departemen Pendidikan Nasional, Sesuai dengan Surat Pelaksanaan Hibah Kompetitif Penelitian untuk Publikasi Internasional , Nomor : 733/H3.13/PPd/2009 , tanggal 13 Juli 2009

UNIVERSITAS AIRLANGGA
DESEMBER 2009

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NASKAH PUBLIKASI

Pinostrobin isolated from *Kaempferia pandurata* Roxb induced apoptosis in TD-47 human beast 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 TD-47 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 andrographolide can induce apoptosis in in TD-47 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 , TD-47 cell , Apoptosis, p53

Introduction

Apoptosis is cellular suicide or programmed cell death which is mediated by activation of an evolutionary conserved intracellular pathway. Recently the relation of apoptosis and cancer has been emphasized and increasing evidence suggests that the processes of neoplastic transformation , progression and metastasis involve alteration of normal apoptotic pathway (Bold *et al.*,1997). Apoptosis also gives some clues about effective anticancer therapy, and many chemotherapeutic agents were reported to exert their anti-tumor effect inducing apoptosis of cancer cells (Kamesaki, 1998). Chemotherapy and radiation are widely used for cancer treatment. Common features of many anti-cancer drugs and radiation are induction of DNA damage followed by activation of the tumour suppressor protein p53 as mediator of their cellular effect. The accumulation and activation of wild-type p53 in at least two pathways, cell cycle arrest and apoptosis. P53-dependent G1 arrest is mediated by direct transvactivation of the $p21^{WAF1}$ gene that encodes the inhibitor of cyclin-dependent kinase (Bates &Vousden,1994).

Kaempferia pandurata Roxb is a traditional medical herb which belongs to the family of Zingibercaceae, shrub grown the moist, shady areas of India, China , Indonesia and throughout Southeast Asia and has been used for treatment of antitussive , antidysentri , diuretic effect , inflammatory effect (Heyne, 1987). According to several phytochemical reports, major constituens of the rhozoma of *Kaempferia pandurata* Roxb are composed flavonoid 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 Methods

Plant material

The 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 Pharmaconosy 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 vacou* 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 recrystalized by hot methanol. Because spectral data of IR, ^{1}H-NMR, ^{13}C-NMR and MS were 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 10^5 unit Penicillin. A humified incubator with 5% CO_2 was used to row the cell at 37°C.

Chemicals

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

Cell morphology

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

Determination of Cell Viability

Cell were plated in triplicate at a density of 1×10^5 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 viabity 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 (Spector *et al.*, 1998). 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 (Spector,1998). Percentage of apoptotic cell was calculated from the formula :

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

Analysis DNA Fragmentation

Cell were treated with 10 , 50 , 100 µg/ml pinostrobin for 24, 48 and 72 hours incubation. Cell was harveseted and suspended in 500 µl of lysis buffer containing 20mM Tris-HCl (pH 7.4), 4 mM EDTA, 0,4%(v/v) Triton X, and incubated on ice 30 min. After centrifugation for 5 min at 13.000 rpm using eppendorf tube centrifuge, supernatant was extracted with phenol three time and once with chloroform . Then DNA was precipitated by incubating at -80°C for 30 min after the addition of 1 µg of glycogen, 100 µl of 5 M NaCl and 700 µl of isopropanol to each sample. DNA was collected by centrifuging at 13.000 rpm for and washed once with 70% ethanol. DNA pellet were dissolved in 30 µl of TE buffer containing 10 µg/ml RNase A, and incubated at 37°C for 30 min. Ten µl of each DNA samples was loaded on 1,8% agorose gel.

Immunohistochemical analysis for p53, bax, bcl-2 and caspase-3

Cell were treated with 10 , 50 , 100 µg/ml pinostrobin for 24, 48 and 72 hours incubation . Cancer cell after treatment were studied by IHC using monoclonal antibodies to p53 ,bax , bcl-2 and caspase-3 (Boeringer Mannheim, Mannheim, Germany). Using methods described previously (Santini *et al.*,1993), the cell were treated using the streptavidin-biotin-peroxidase complex method with the following modifications. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min. Sections were blocked with 20% horse serum for 1 h to prevent nonspecific binding. Primary antibody at a 1:50 dilution was applied overnight at 4°C, with appropriate negative and positive controls. Secondary biotinylated antibody at 1:200 was applied for 1 h to visualize bound antibody. SABC reaction was performed for 1 h at room temperature. The peroxidase activity was developed by incubation in 0.05% 3,3'-diaminobenzidine for 5 min, and slides were counterstained with hematoxylin. Immunoreactive score was determined semiquantitatively by means of a visual grading system in which staining intensity was categorized as grade by assessing the following categories: 0 (0–4%), 1 (5–24%), 2 (25 – 49%), 3 (50 – 74%), or 4 (75 – 100%). according to the previously reported criteria (Katja *et al.*,1999).

Statistical analysis

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

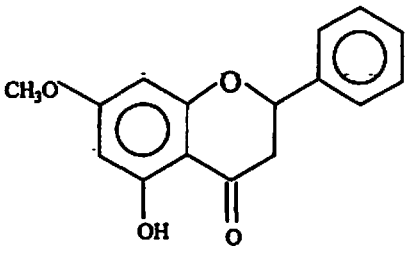
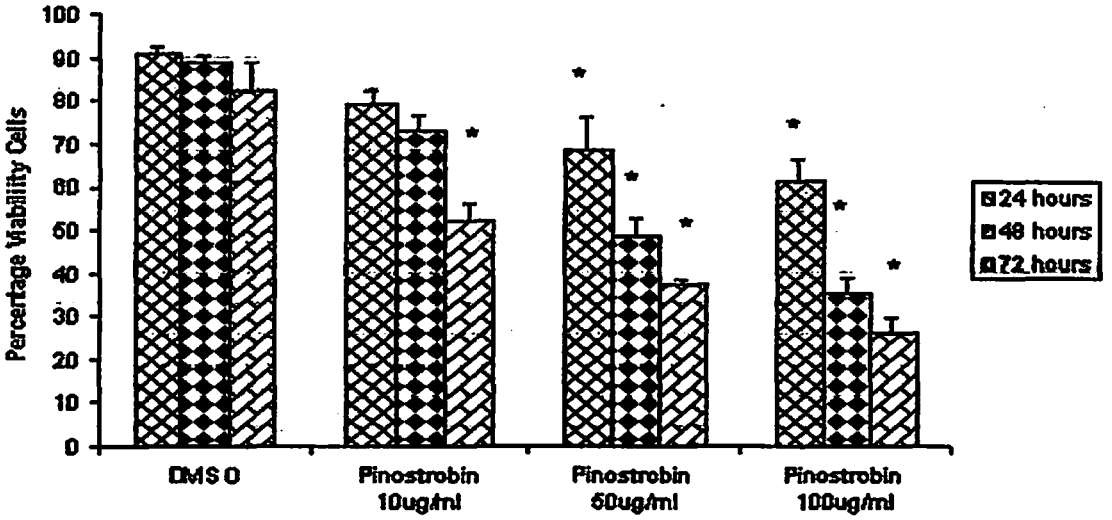


Fig 1. Structural formula of pinostrobin

Results and discussion

The inducting apoptosis activity of pinostrobin were evaluated using many other methods , such as cell morphology , histochemical staining by acridine orange – ethidium bromide , DNA fragmentation analysis and immunohistochemical to analysis expression of tumor suppression protein p53, bcl-2 family protein pro- anti antiapoptosis such as Bax and bcl2 , and also analysis caspasse-3 . The methods are used to detect the morphologic changes associated with apoptosis include chromatin condentation and pyknosis, fragmentation of nucleus and formation of apoptotic bodies of membrane – enclosed pieces of condensed chromatin and well-preserved organelle (Spector, 1998 ; Arend *et al.*, 1990). Another to elucidate further the mechanism of pinostrobin - induced apoptosis, we investigated a potential role for the p53 pathway or instrinsic pathway of apoptosis. Because the pinostrobin exhibited Topoisomerase I inhibitor activity (Sukardiman *et al*, 2000) , and the stabilization of topoisomerase II-DNA cleavable complexes, rather than the inhibition of topoisomerase catalytic activity, is essential for drug cytotoxicity effect in tumour cell (Pommier [a], 1996 ; Pommier [b],1997 ; Chen & Liu , 1994). The cleavable complexes induced by topoisomerase I inhibitors are only potentially cytotoxic, since the complexes are readily reversible upon drug depletion (Pommier [a], 1996 ; Pommier [b],1997; Chen & Liu , 1994). However, when the cleavable complexes persist, permanent DNA damage occurs, resulting in cell death mainly by apoptosis, we suggested pinostrobin - induced apoptosis followed intrinsic pathway via activation p53 and proapoptotic bax.



with control DMSO ; at 10 , 50 , 100 µg/ml pinostrobin 24 , 48 and 72 h, and cell viability was determined by trypan blue exclusion . Data represent mean ± SEM of triplicate. * $p < 0.05$ compared to control (One Way ANOVA followed by Tukey's multiple comparison test).

Pinostrobin (fig.1) showed a significant inhibitory effect on the growth and viability of TD-47 human breast cancer cell in vitro. The inhibitory action was noticed to be concentration and time dependent. The maximum inhibitory effect of andrographolide was obtained at concentration of 100 µg/ml pinostrobin at 72h, where their percentage cell viability were 0.28% respectively. On the other hand , after 0.35mM pinostrobin and treatment at 24h , percentage cell viability were 71.45% respectively and significantly decreased after 48 and 72h , percentage cell viability were 44.83% and 23.52% respectively. After 0.75mM pinostrobin treatment at 24h , percentage cell viability were 54.45% respectively and significantly decreased after 48 and 72h, percentage cell viability were 9.45% and 2.5% respectively. On the other hand , after 1.40mM pinostrobin treatment at 24h , percentage cell viability were 34.35% respectively and significantly decreased after 48 and 72hour , percentage cell viability were 4.36% and 0.28 % respectively, as show in fig (2).

When TD-47 human breast cancer cell was treated with various concentration of pinostrobin for 48 h, were examined under a phase contrast microscope they exhibited distinct morphological features of apoptosis, such as cellular shrinkage and formation of apoptotic bodies , as show in fig (3).

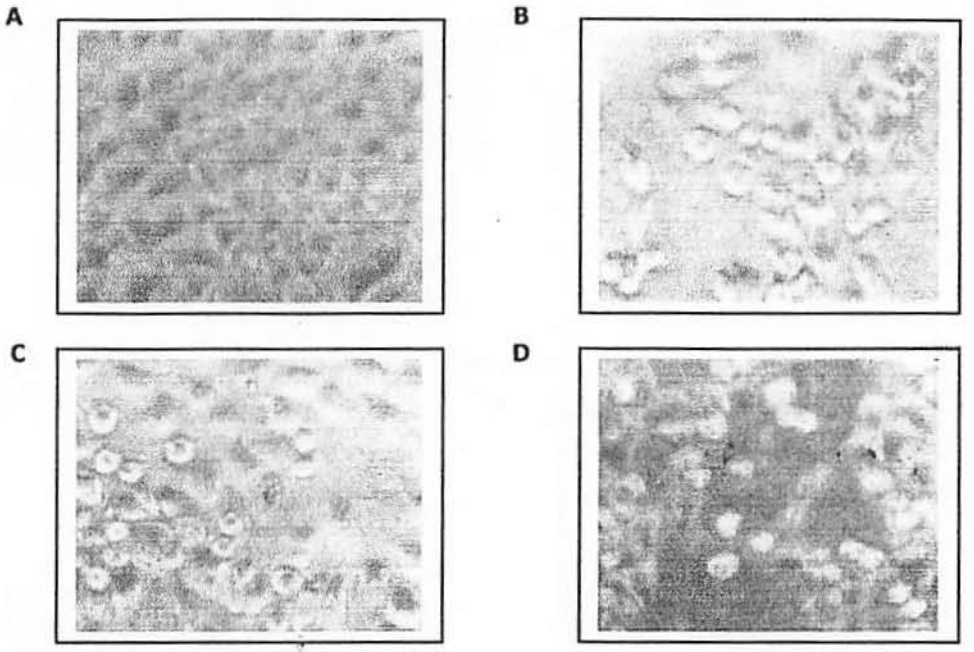


Fig. 3. Effect of pinostrobin on morphology change of TD-47 human breast cancer cells (phase-contrast microscopy). A, untreated cells cultured (DMSO 0,1%). B. cells were treated with 10µg/ml pinostrobin . C cells were treated with 50 µg/ml pinostrobin D. cells were treated with 100 µg/ml pinostrobin for 48 h.

After 24 hour treatment of TD-47 human breast cancer cell with 10 , 50 , 100 µg/ml pinostrobin , nuclear structure using Acridine Orange / Ethidium Bromide (AO / EB) staining , examained using flourecent microscopy, exhibited condentation and fragmentation of some nuclei, as shown in fig.(4). Live cell will appear uniformly green.. Early apoptotic cell will stain green an contain bright green dots in the nuclei as a consequence chromatin condensation and nuclear fragmentation. Late apoptotic cell will show condensed and often fragmented nuclei and also incorporate ethidium bromide and there for stain orange (Spector, 1998).

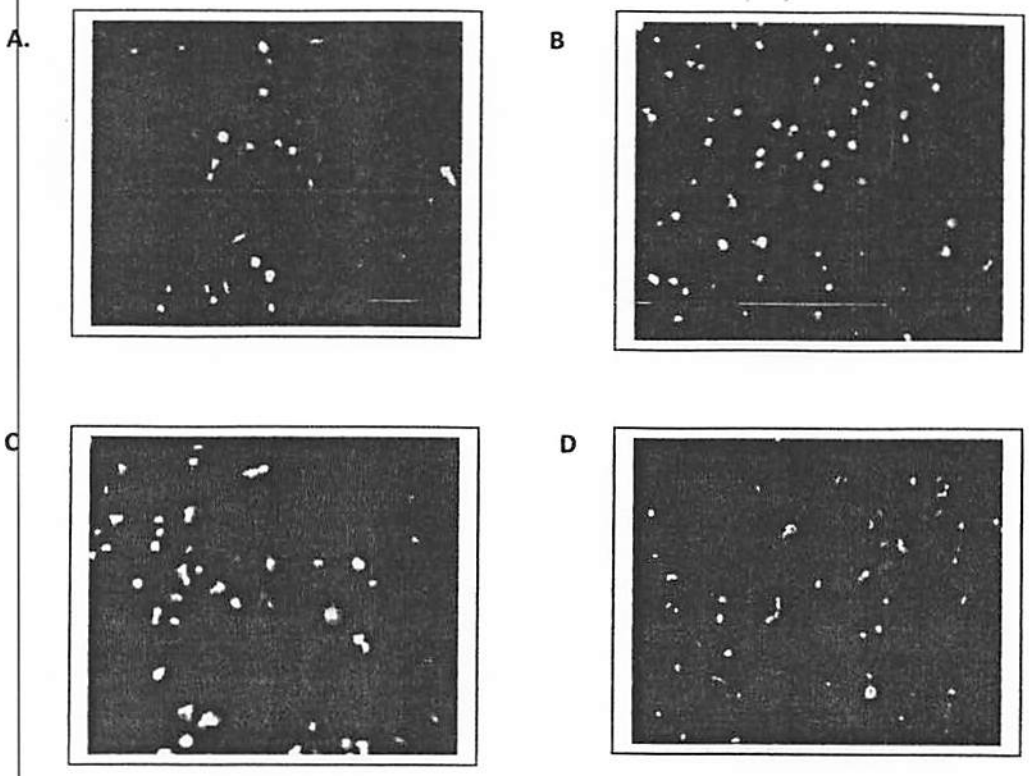


Fig. 4. Effect of pinostrobin on induction apoptosis of T47-D human breast cancer cells , analysis by Acridine Orange / Ethidium Bromide (AO / EB) staining (examained using flourecent microscopy). A, untreated cells cultured (DMSO 0,1%). B. cells were treated with 10 μg/ml . C cells were treated with 50 μg/ml D. cells were treated with 100 μg/ml pinostrobin for 24 h.

After 24, 48 and 72 hour treatment of TD-47 human breast cancer cell with 10 , 50 , 100 μg/ml pinostrobin , nuclear structure using acridine orange / ethidium bromide (AO / EB) staining , examained using flourecent microscopy, exhibited the percentage of apoptotic cell increased in time- concentration – related manner , as show in fig (5).

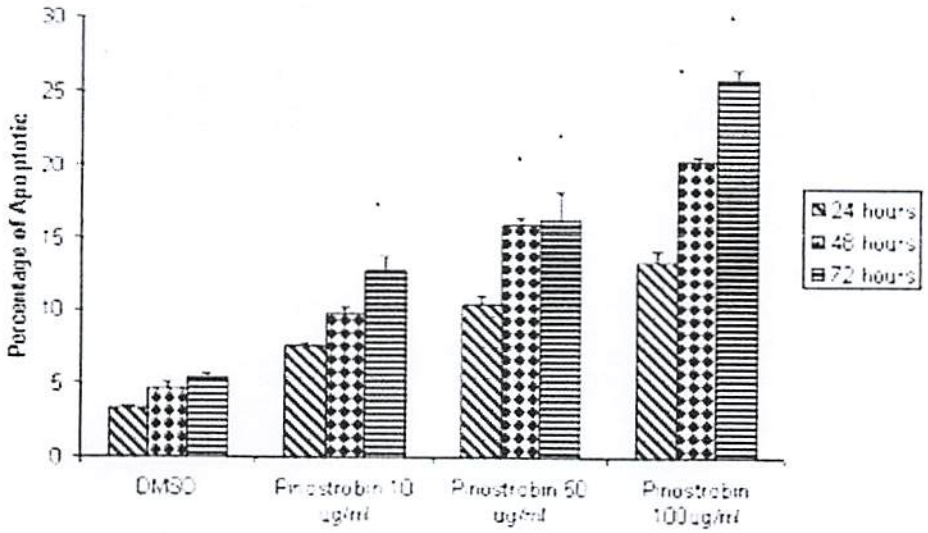
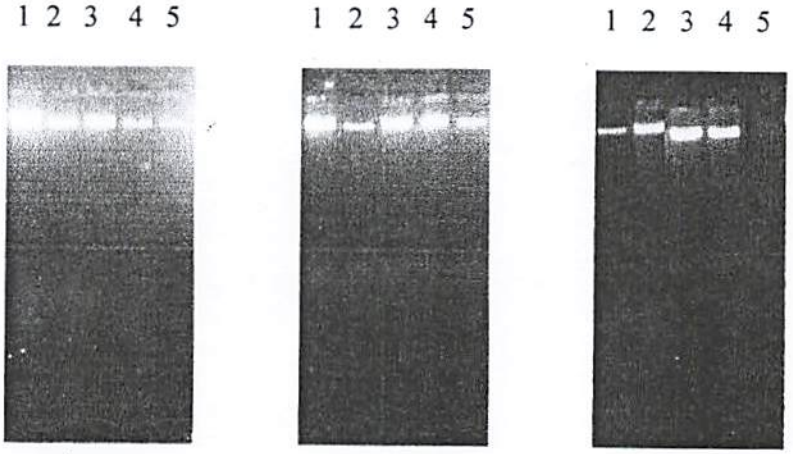


Figure 5. Relative apoptosis induced by pinostrobin as determined by Acridine Orange - Ethidium Bromide (AO / EB) staining . The percentage apoptotic cell was examined for each case and the result expressed as number of apoptotic cells over total number of cell counted . Data represent mean ± SEM of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

After 72 h treatment of TD-47 human breast cancer cell with concentration 100 µg /ml pinostrobin it was found that it induced internucleosomal DNA fragmentation , one of biochemical hall mark of apoptosis, at concentration , as show in fig (6). Comparable finding were reported in the study Chopin *et al.*, (2002) where they did not found internucleosomal DNA fragmentation from TD-47 human breast cancer cell when treatment with 2.5mM sodium butyrate for 48h.



(A) 24 hours (B) 48 hours (C) 72 hours

LAMPIRAN 2
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Re: Submission article

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Thank for your attention,

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LAMPIRAN 1
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expression of Bcl-2 family members by andrographolide treatment may trigger the activation of initiator caspases 8 and 9 followed by activation of effector caspases 3 and 6.

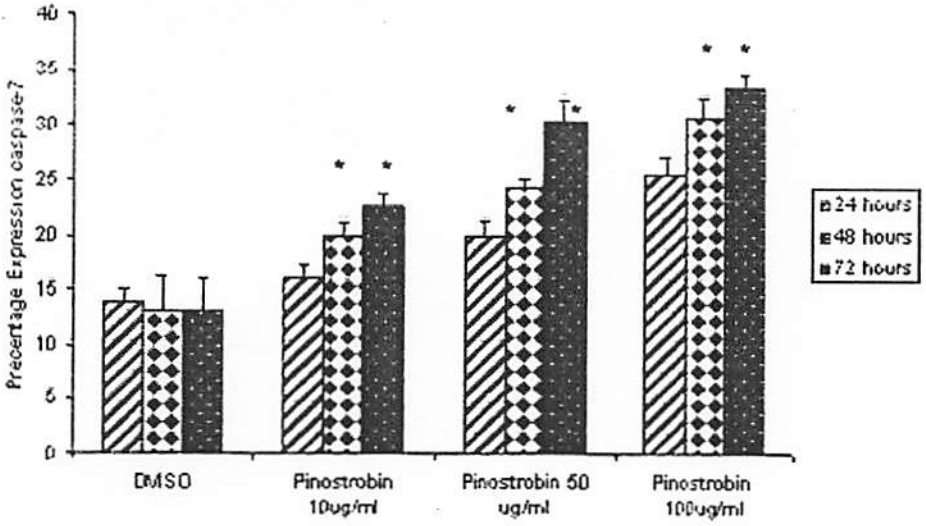

Figure 10. Concentration course experiment for pinostrobin - induction increased expression of caspase-7 from T47-D human breast cancer cells , analysis by immunohistochemical.. Data represent mean ± SEM of triplicate. * $p < 0.05$ compared to control (One Way ANOVA followed by Tukey's multiple comparison test).

These abilities of andrographolide to induce apoptosis implies its potential as chemotherapeutic agent because many anticancer drugs are known to achieve their antitumor function by inducing apoptosis in tumor cells. Although the precise molecular mechanism by which apoptosis is induced by pinostrobin remains unclear, it might be a potent useful antitumor agent against breast cancer.

Acknowledgments

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Reference

Bail,L., Aubourg L., Habrioux G., 2000. Effect of pinostrobin on estrogen metabolism and estrogen reseptor transactivivation, **Cancer Lett**, Aug, 1;156910;37-44.

Figure 8. Concentration course experiment for pinostrobin - induction increased expression of bax from T47-D 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).

When TD-47 human breast cancer cell was treated with various concentration of pinostrobin from *Kaempferia pandurata* Roxb for 24h, 48h and 72h , the expression bcl-2 decreased in concentration – related manner analysis by Immunohistochemical method , as show in fig (9).

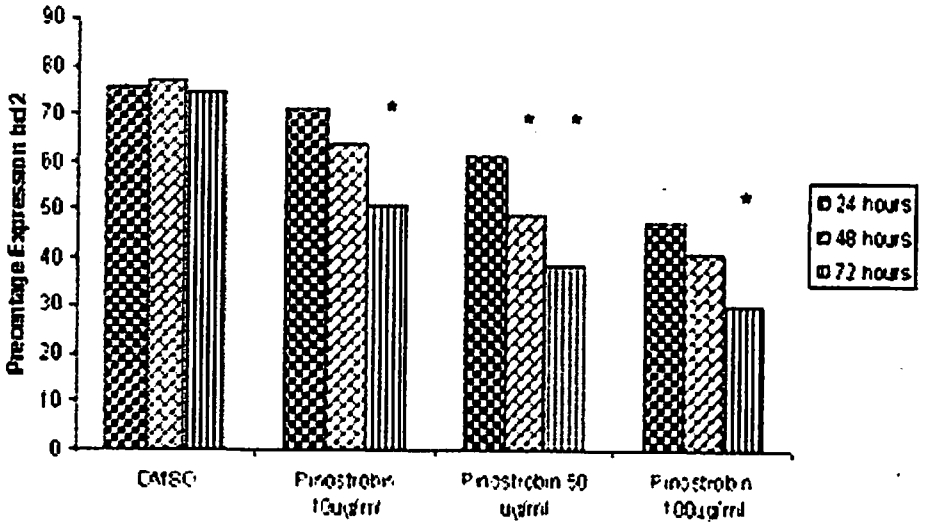

Figure 9. Concentration course experiment for pinostrobin - induction decreased expression of bcl-2 from T47-D human breast cancer cells , analysis by Immunohistochemical.. Data represent mean ± SEM of triplicate. * p < 0.05 compared to control (One Way ANOVA followed by Tukey's multiple comparison test).

After TD-47 human breast cancer cell was treated with various concentration of pinostrobin from *Kaempferia pandurata* Roxb for 24h, 48h and 72h , the expression caspase-3 increased in concentration – related manner analysis by immunohistochemical metdhod , as show in fig (10).

In this study, pinostrobin treatment to TD-47 human breast cancer cells led to a remarkable induction of apoptotic cells. Pinostrobin treatment also led to decline viability cell , morphological change , nucleosomal DNA fragmentation , activation of p53 expression , increased bax , decreased bcl-2 and increased caspase-7 The ratio of Bax:Bcl-2 is critical to cell survival such that an increase in Bax levels can shift the process in favor of apoptosis (Pepper *et al.*, 1997). Andrographolide induced apoptosis and cell growth inhibition was accompanied with decrease in Bcl-2 with concomitant increase in Bax in cell . This altered

Fig. 6. Concentration course experiment for pinostrobin - induction internucleosomal DNA fragmentation T47-D 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 .

When TD-47 human breast cancer cell was treated with various concentration of pinostrobin from *Kaempferia pandurata* Roxb for 24h, 48h and 72h , the expression p53 increased in time and concentration – related manner analysis by immunohistochemical method , as show in fig (7).

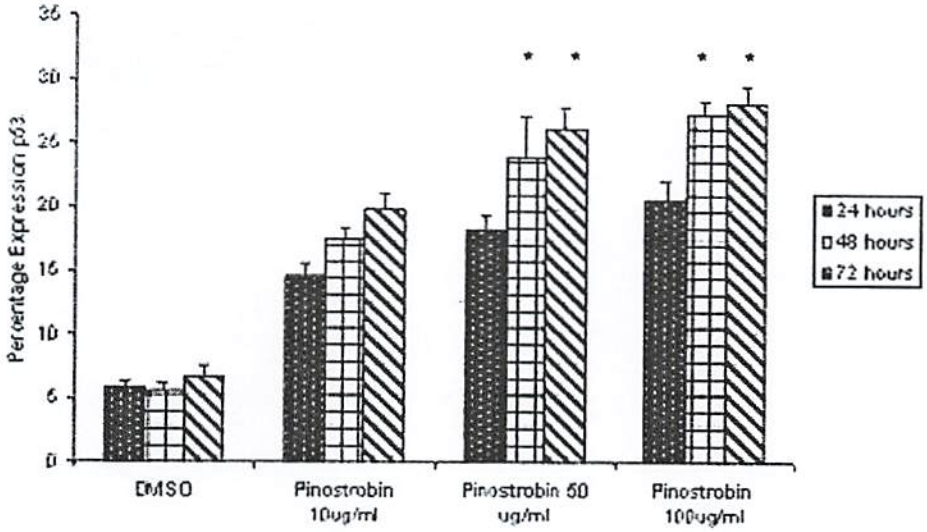

Figure 7. Concentration course experiment for pinostrobin - induction increased expression of p53 from T47-D human breast cancer cells , analysis by immunohistochemical.. Data represent mean ± SEM of triplicate. * $p < 0.05$ compared to control (One Way ANOVA followed by Tukey's multiple comparison test).

After TD-47 human breast cancer cell was treated with various concentration of pinostrobin from *Kaempferia pandurata* Roxb for 24h, 48h and 72h , the expression bax increased in concentration – related manner analysis by immunohistochemical method , as show in fig (8).