CHAPTER IV

RESULTS

1. Evaluation of cytotoxic effect of DF-A on H460 cells and HaCat cells

The cytotoxic effect of the compound on human lung cancer and human normal keratinocyte cells was first characterized. Cells were treated with various concentrations of DF-A (0-100 μM) for 24 h and cell viability was measured by MTT assay. Figure 19 shows that DF-A significantly decreases cell viability of H460 cells at the concentrations greater than 10 μM. For HaCat cells, DF-A caused significant toxic effect at the concentrations greater than 50 μM. Therefore, DF-A at the concentrations of 0-5 μM were considered non-toxic to both cells.

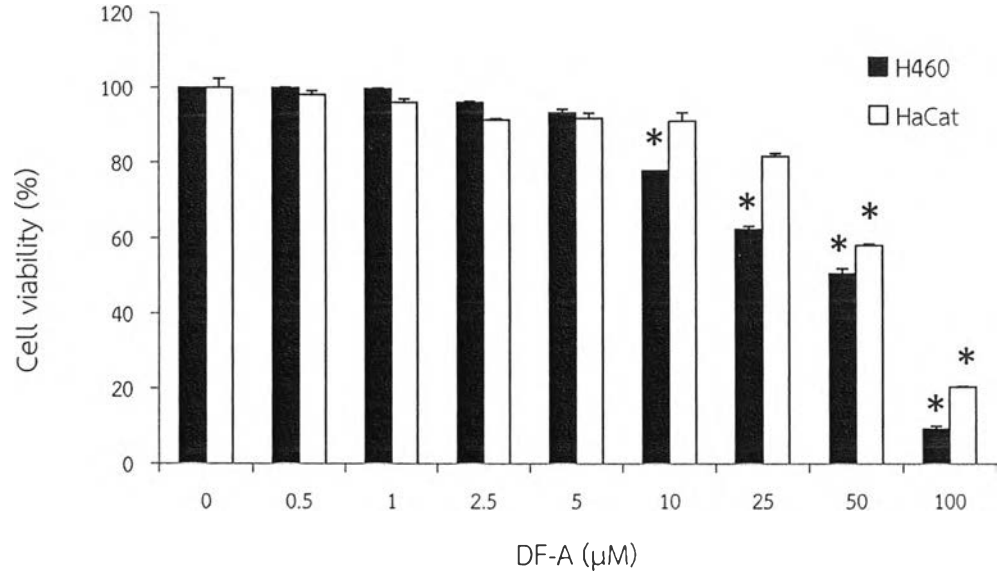
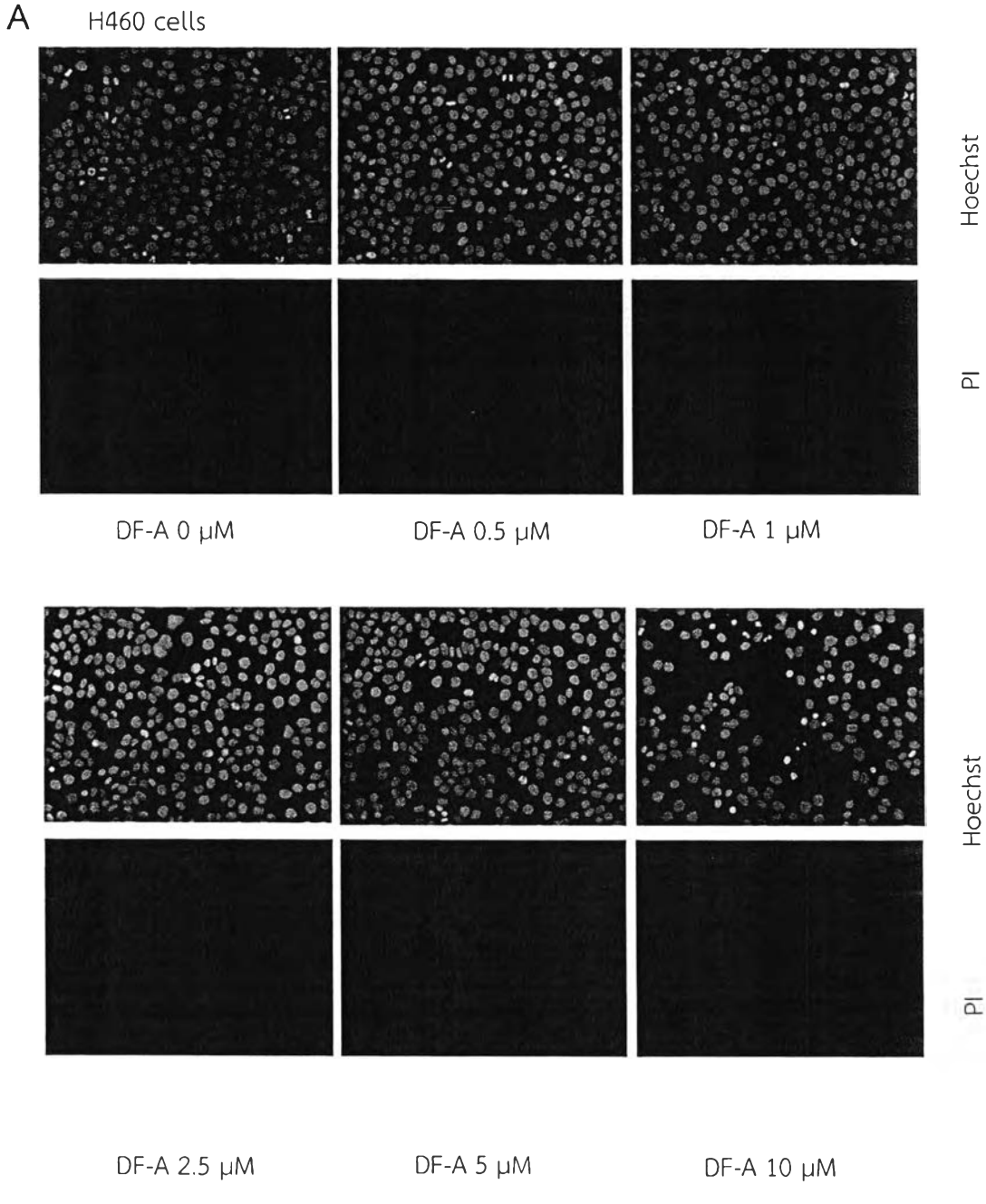
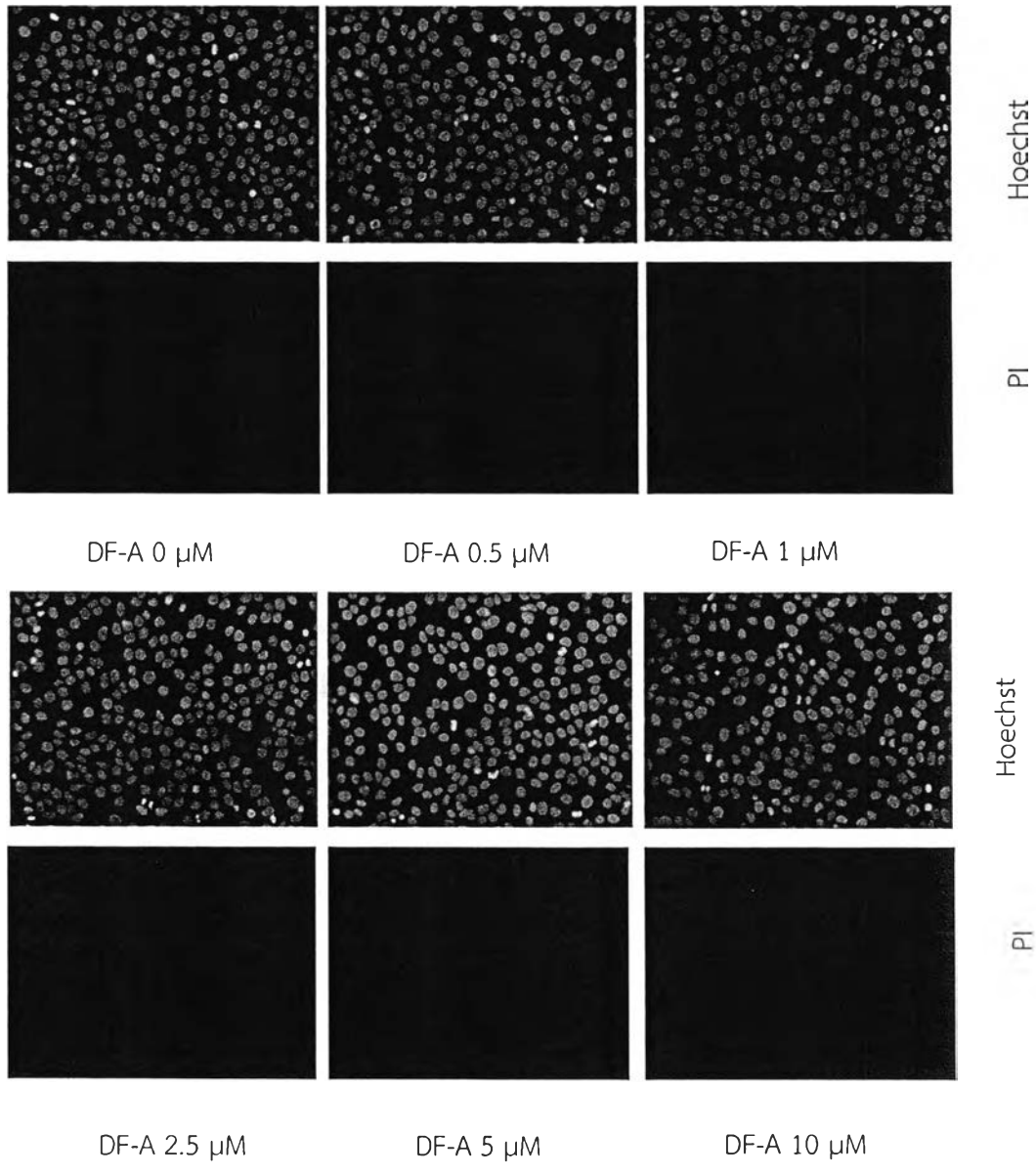


Figure 19 The cytotoxic effects of DF-A on lung cancer H460 cells and keratinocyte HaCat cells. Cells are treated with DF-A (0-100 μM) for 24 h. Percentage of cell viability determined by MTT assay. Values are means of 3 independent triplicate experiments ± SE. * $p<0.05$ versus non-treated control.

In order to confirm the above results, the effect of DF-A on apoptosis of cells was evaluated by Hoechst33342/PI co-staining assay. The nuclear morphology analysis indicated that DF-A at the concentrations of 0-5 μM caused no significant apoptosis indicated by the absence of condensed and/or fragmented nuclei (Figure 20).



B HaCat cells



C

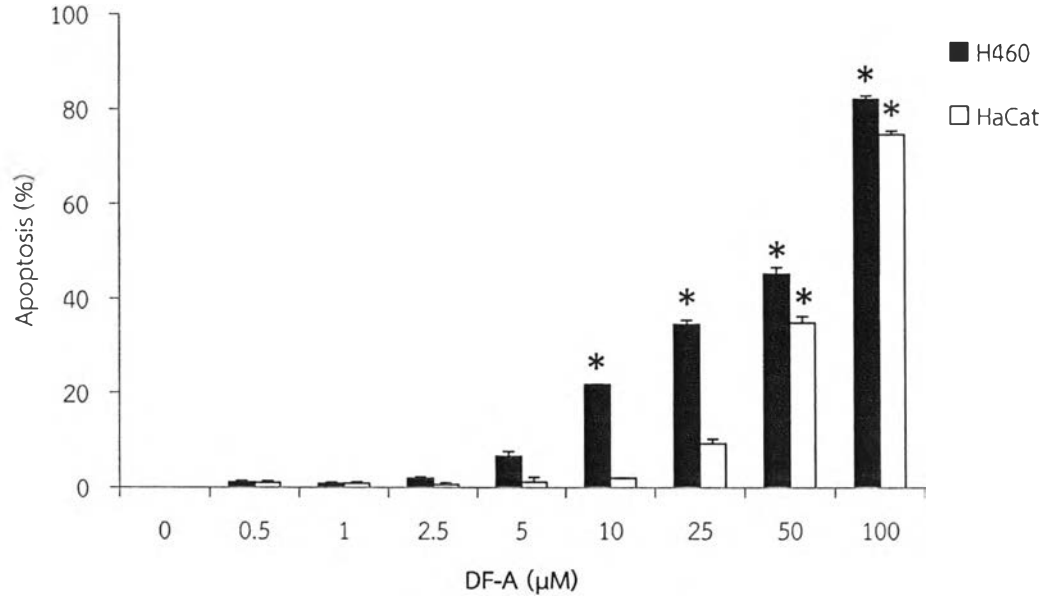


Figure 20 Nuclear morphology of the cells detected by Hoechst 33342/PI co-staining and visualized under a fluorescence microscope. (A) Nuclear morphology of H460 cells. (B) Nuclear morphology of HaCat cells. (C) Percentage of apoptotic cells detected by Hoechst33342 staining on lung cancer H460 cells and keratinocyte HaCat cells. Cells are treated with DF-A (0-100 μM) for 24 h. Values are means of 3 independent triplicate experiments ± SE. * $p<0.05$ versus non-treated control.

2. Anoikis effect of DF-A on H460 cells and Hacat cells.

Because anoikis is accepted as an important mechanism in inhibition of cancer metastasis, compounds that significantly increase anoikis response are of interest for further development for anti-metastasis approaches (112). To determine the anoikis-sensitization activity of DF-A, H460 cells were detached and suspended in culture media containing DF-A (0 – 5 μM) in non-adhesive poly-HEMA coated-plates for 0-24 h, and cell survival was determined by XTT assay. Figure 21 shows that the cell survival was gradually decreased over time and approximately 60 % of the cell remained survive at 24 h after cell detachment whereas such anoikis sensitization effect was not detectable in HaCat cells (Figure 22). DF-A significantly reduced cell survival in a dose-dependent manner. The significant decrease of cell survival was detectable as early as 6 h after treatment with 5 μM DF-A.

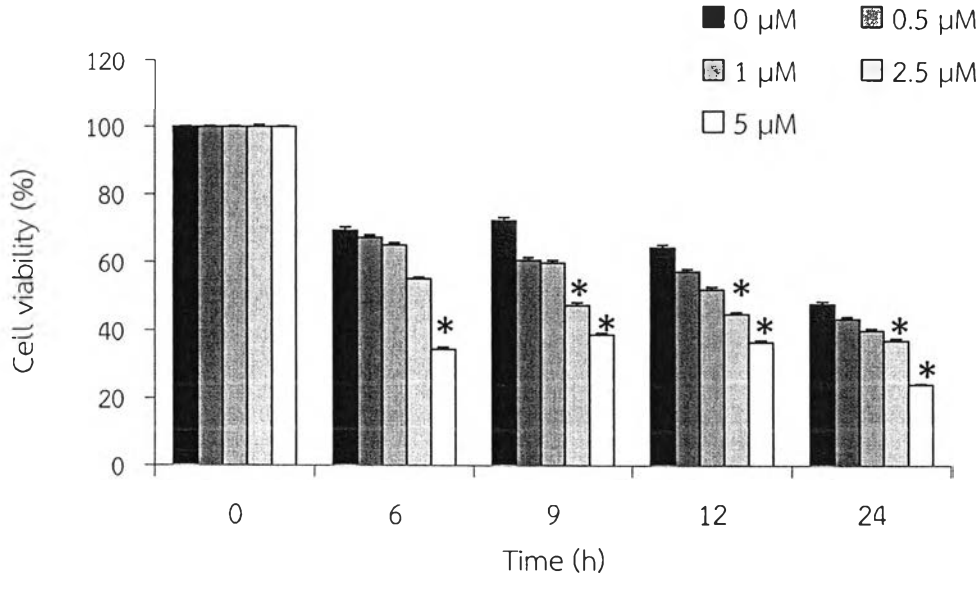


Figure 21 Anoikis sensitizing effect of DF-A. Detached H460 cells were incubated with DF-A (0-5 μM) in poly-HEMA coated plates. Cell viability was determined by XTT

assay at indicated times. Values are means of 3 independent triplicate experiments ± SE. *p<0.05 versus non-treated control at time 0

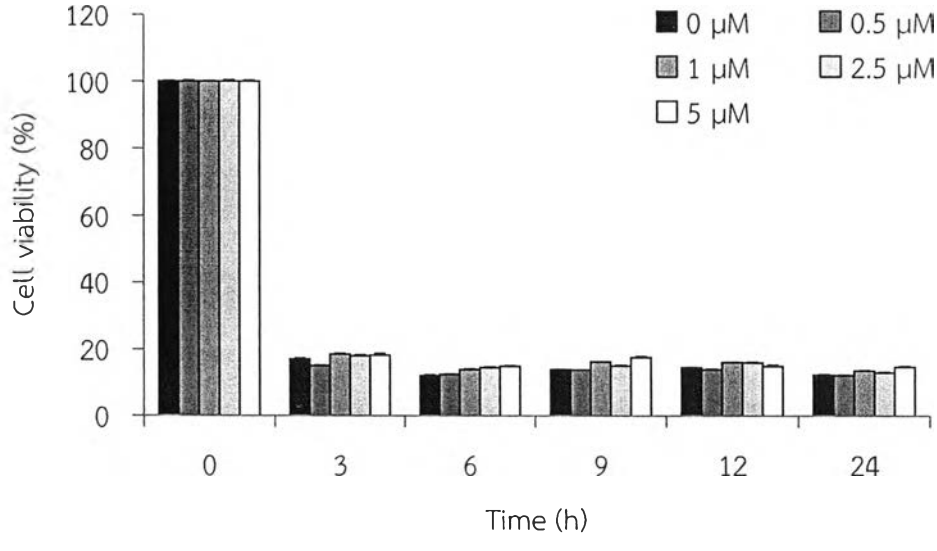


Figure 22 Anoikis sensitizing effect of DF-A. Cell viability of detached HaCat cells after cells were treated with DF-A (0-5 μM) in poly-HEMA coated plates. Values are means of 3 independent triplicate experiments ± SE. *p<0.05 versus non-treated control at time 0.

Apoptosis evaluation revealed that DF-A significantly increase the anoikis cells in a dose-dependent manner with 80% of the detached cells treated with 5 μM DF-A showing apoptotic characteristic (Figures 23 and 24). The percentage of apoptotic cells was significantly increased in response to DF-A at concentration ranging from 2.5 μM to 5 μM, whereas PI-positive cell was not detectable in such a condition.

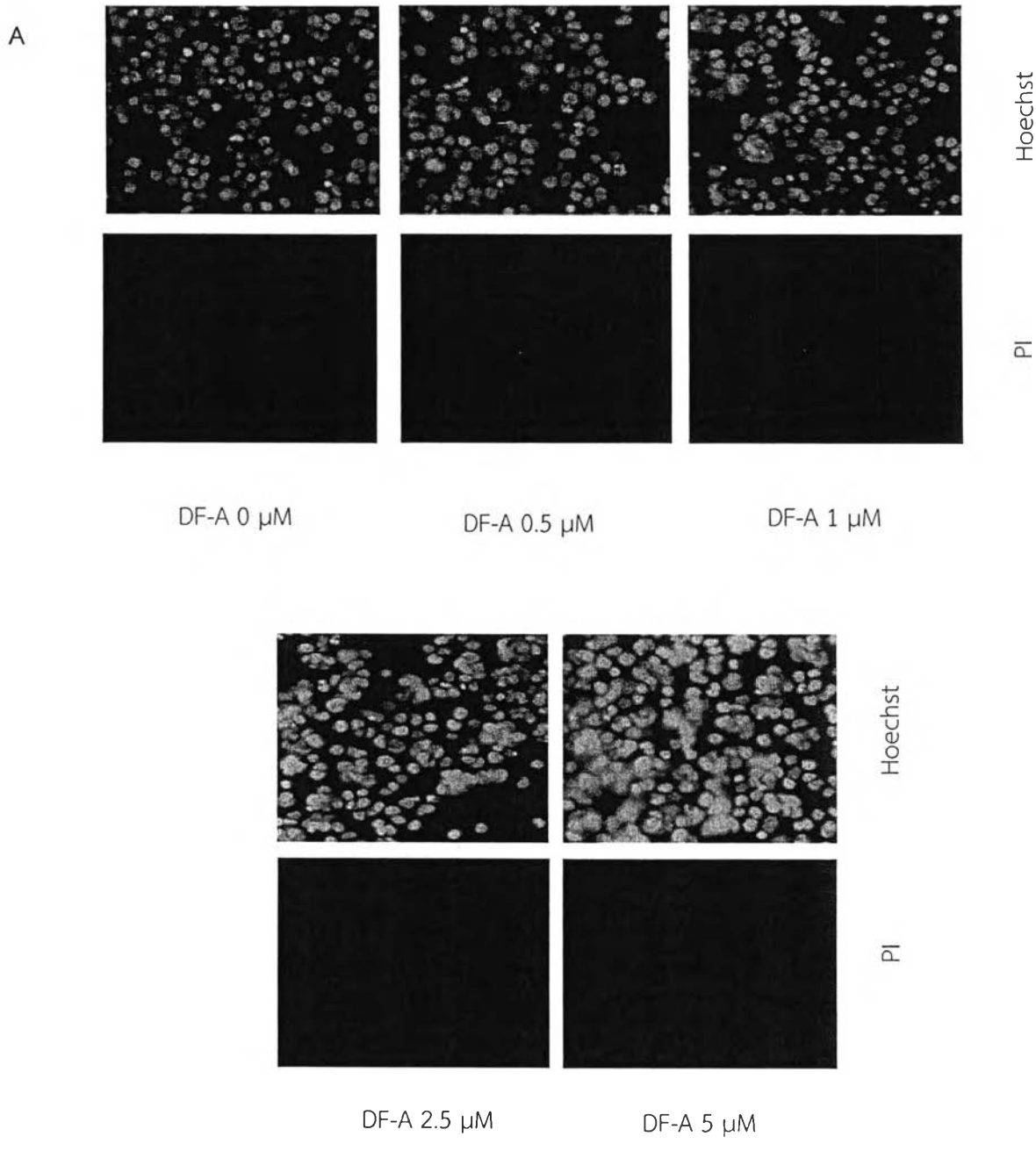


Figure 23 Morphology of anoikis nuclei stained of H460 cells with Hoechst 33342/PI for 24 h.

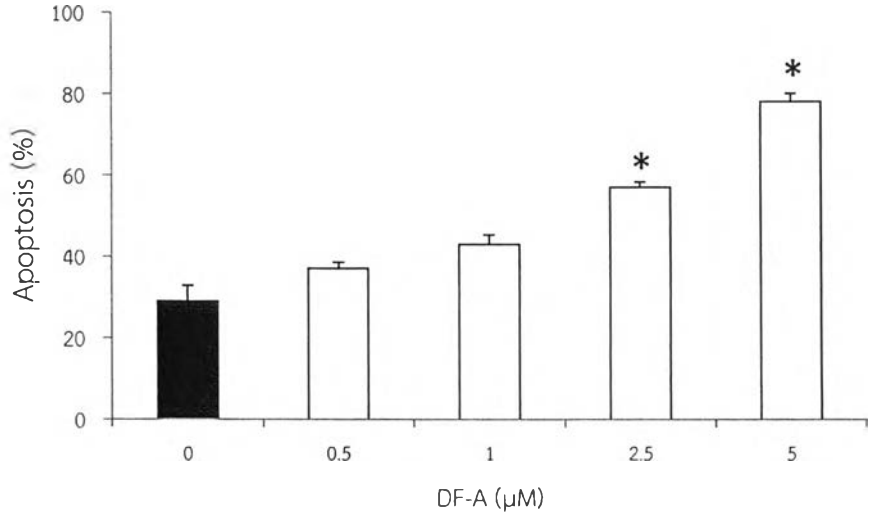
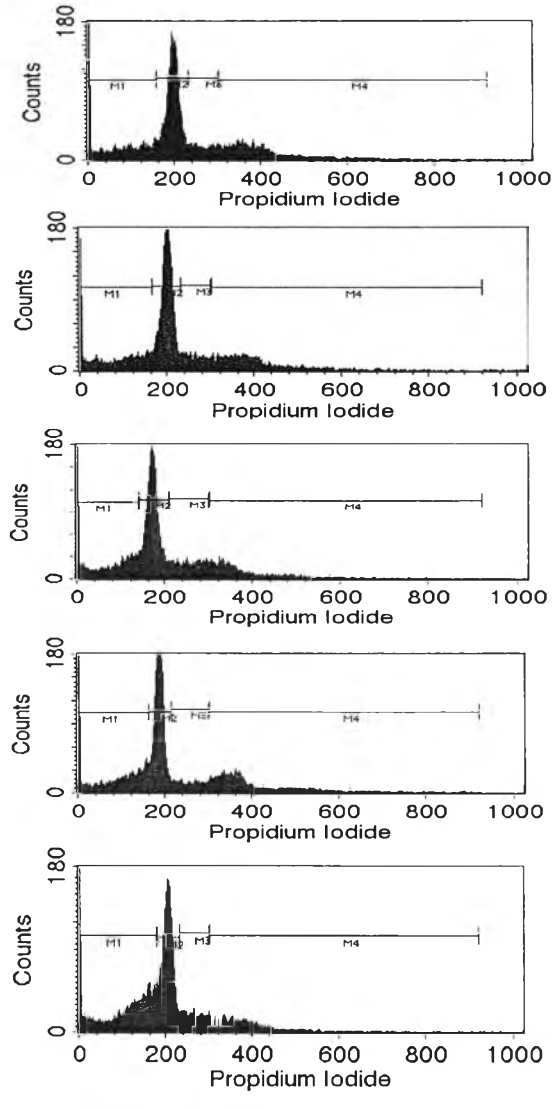


Figure 24 Percentage of apoptotic cells in response to DF-A treatment. Values are means of 3 independent triplicate experiments ± SE. *p<0.05 versus non-treated control at time 0.

Furthermore, we analyzed sub-G_0 fraction of the cells treated with DF-A using flow cytometric analysis. Results indicated that the treatments of the cells with DF-A significantly increased the sub-G_0 fraction in a dose-dependent manner (Figures 25(A) and 25(B)). Together, these results demonstrated herein that DF-A at non-toxic concentrations significantly sensitized the lung cancer cells to anoikis.

A



DF-A 0 μM

DF-A 0.5 μM

DF-A 1 μM

DF-A 2.5 μM

DF-A 5 μM

B

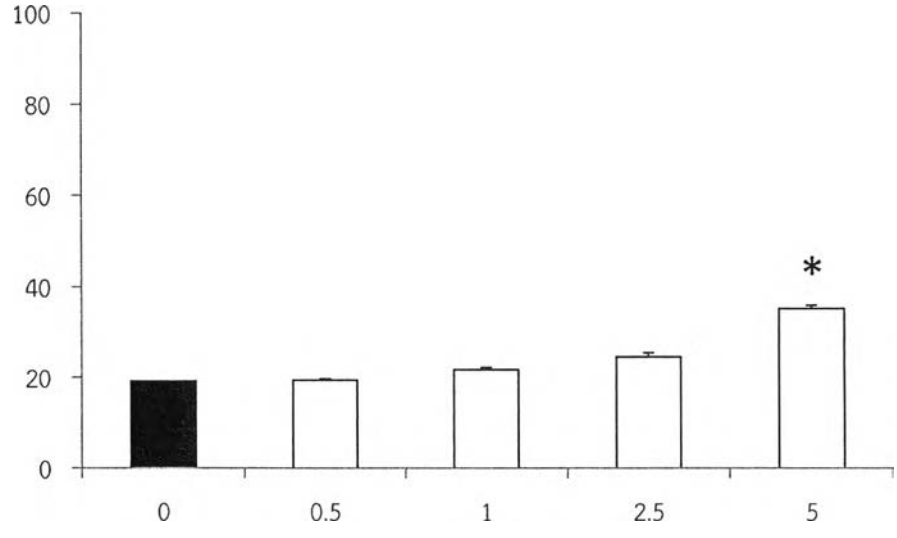


Figure 25 (A) The percentage of sub-G_0 fraction of DF-A treated cells was determined by PI staining and flow cytometry. (B) Relative Sub-G_0 fraction of DF-A treated or untreated cells. Values are means of 3 independent triplicate experiments ± SE. *$p<0.05$ versus non-treated control

3. DF-A inhibits anchorage-independent growth of H460 cells

The growth in anchorage-independent condition was shown to be tightly related to aggressiveness as well as metastasis potential of cancer cells (5, 11). Having shown that DF-A could sensitize H460 cells to anoikis, we further tested whether the compound inhibits growth of the cells in anchorage-independent condition. H460 cells were cultured in the absence or presence of DF-A in anchorage-independent growth condition for 2 weeks, and number and size of cell colony were determined as described. Figure 27(A) shows that treatment of the cells with 1-5 μM DF-A significantly decrease the colony size in comparison to that of non-

treated control cells. The number of colony was found to be significantly reduced in response to DF-A at the concentrations of 2.5-5 μM (Figure 27(B)). Clearly, our findings indicated the ability of DF-A in inhibition of tumor cell growth in anchorage-independent condition. These results supported the potential of the compound for anti-metastasis approaches.

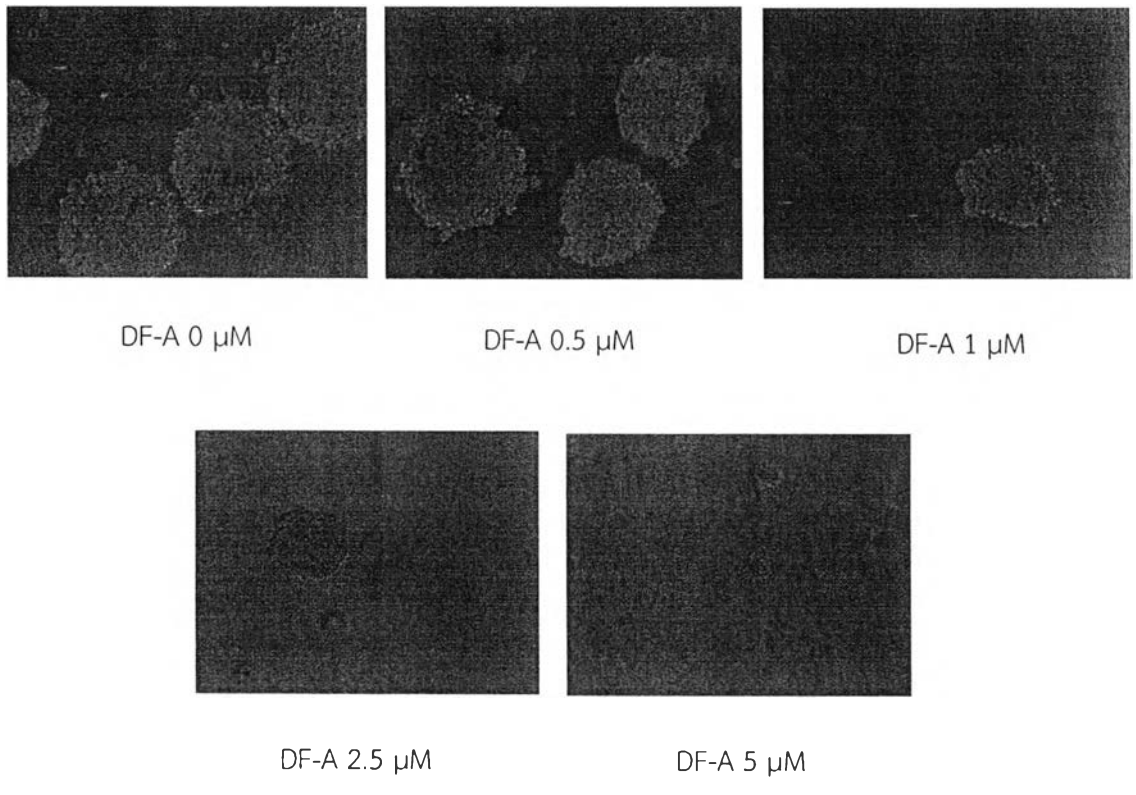


Figure 26 DF-A inhibits anchorage-independent growth. Cells were subjected to soft agar colony formation assay as described in Materials and Methods. Representative fields from three independent experiments were photographed.

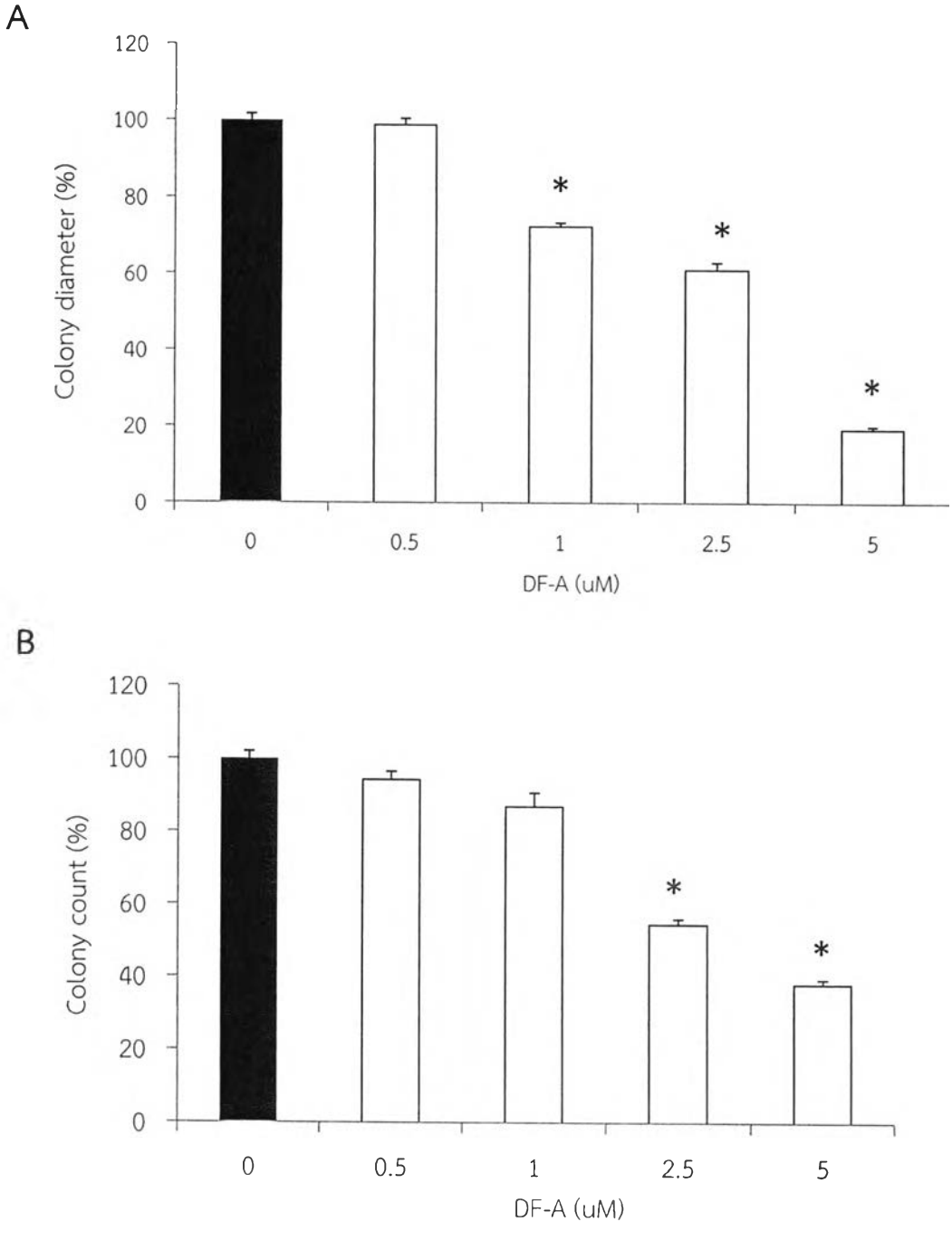


Figure 27 Colony size and number were determined by image analyzer. Values are means of 3 independent triplicate experiments ± SE. *$p<0.05$ versus non-treated control.

4. DF-A decreases anti-apoptotic, survival related, and Caveolin-1 proteins.

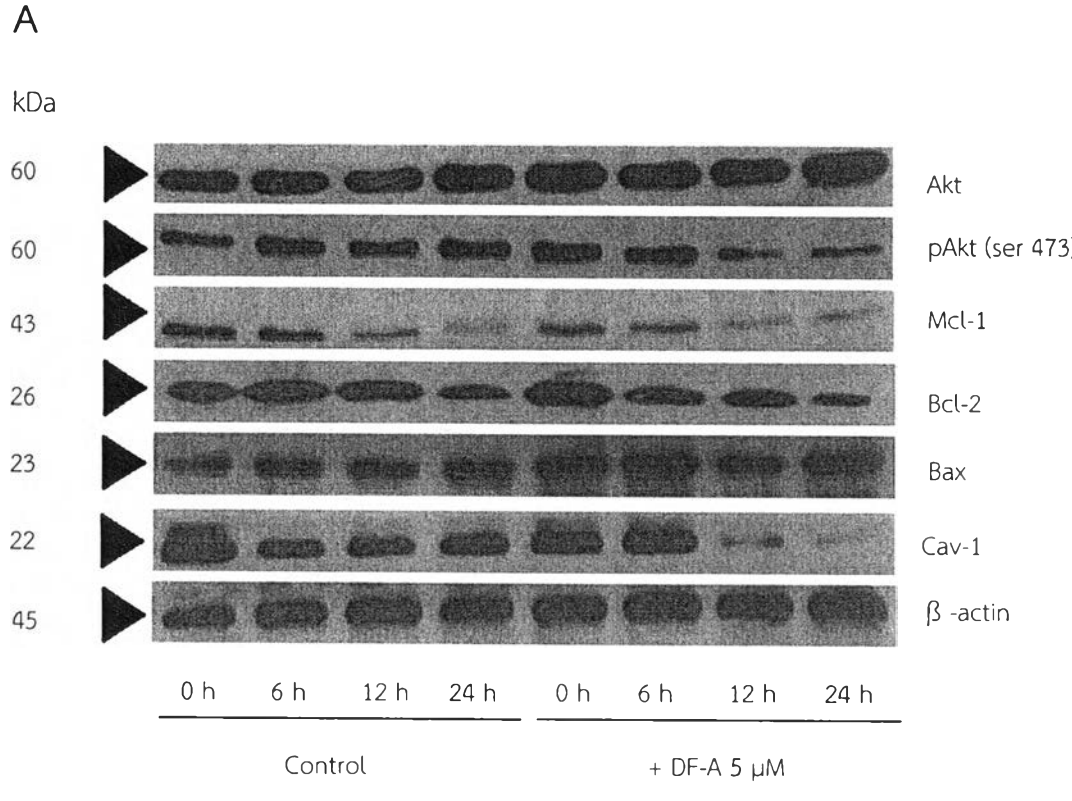
To clarify the mechanisms of DF-A compound in regulation of anoikis in these cells, effects of compound on proteins regulating anoikis of the cells, namely, activated Akt, Mcl-1, Bcl-2, Bax, and Cav-1 were determined.

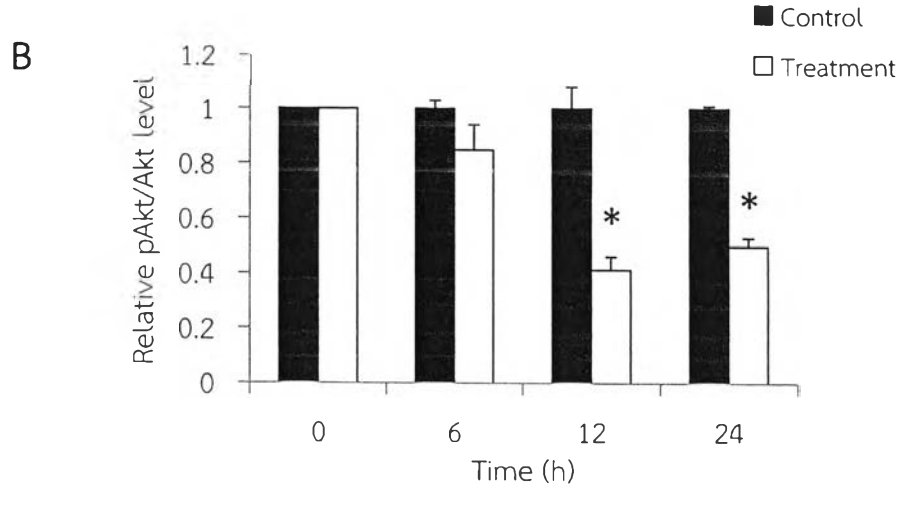
Firstly, the protein alterations in response to cell detachment were clarified. In the absence of DF-A treatment, activated Akt (Phosphorylated Akt Ser 473), total Akt, Mcl-1, Bcl-2, Bax, and Cav-1 levels were determined over time. Results indicated that the decrease of Cav-1, Mcl-1 and Bcl-2 proteins was observed at 6, 12, and 24 h after detachment, respectively. Pro-apoptotic Bax protein was found to increase as early as 6 h after cell detachment. These results suggested that the alteration of the balance between anti- and pro-apoptotic members of Bcl-2 family proteins is a critical caused of anoikis in this condition.

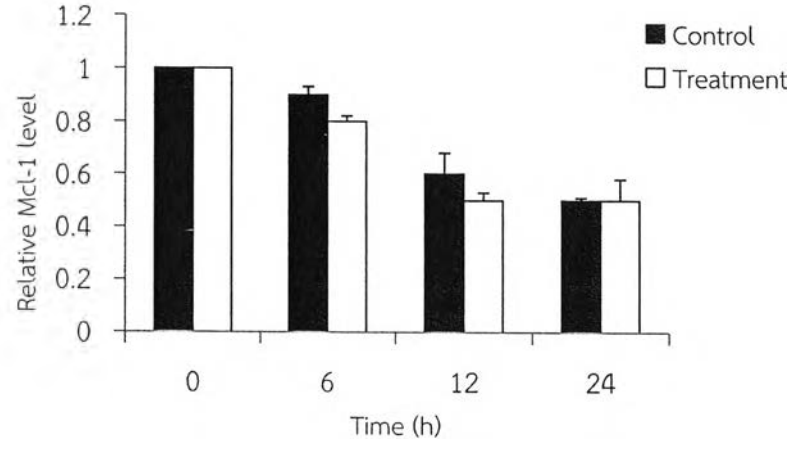
To investigate the mechanism of anoikis sensitization mediated by DF-A, the non-adhesive H460 cells were treated with the 5 μM DF-A for indicated times, and the expression levels of the named proteins were evaluated by western blot analysis. Figure 28 shows that treatment with DF-A significantly down-regulated the activated Akt level in comparison to that of non-treated control, while the total Akt was not affected. Also, the anti-apoptotic Bcl-2 proteins were dramatically reduced as early as 6 h after treatment. It is worthy noted that the patterns of anti-apoptotic Mcl-1 and Bax were only slightly affected by DF-A treatment.

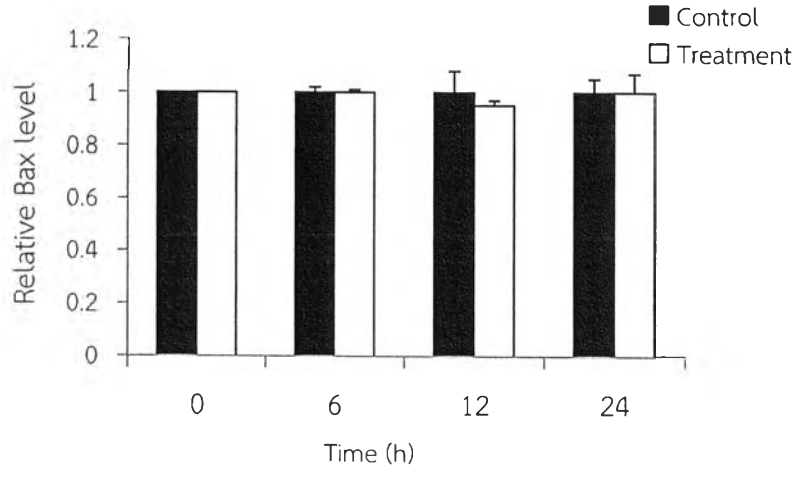
Because the up-regulation of Cav-1 protein is associated with anoikis resistance in many cancers including lung cancer (19, 20), the effect of DF-A on Cav-1 expression was evaluated. As shown in figure 28, the level of Cav-1 was significantly down-regulated in response to DF-A treatment. It is widely accepted that the anoikis

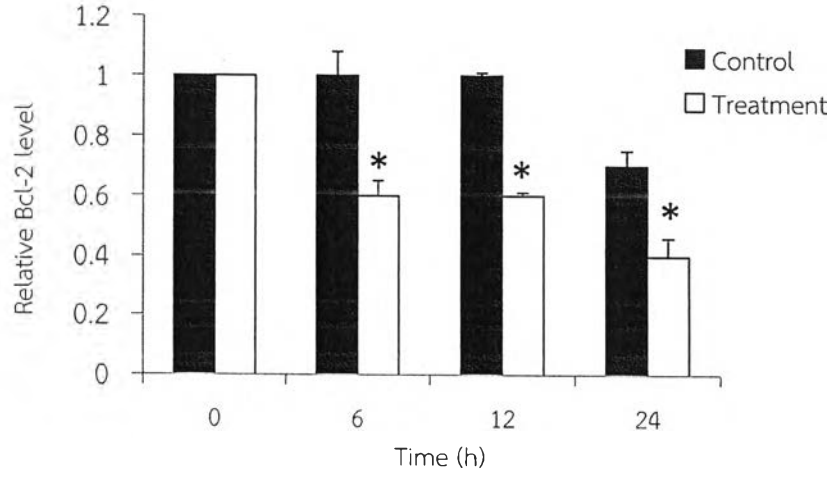
resistance mechanisms of cancer cells involves multiple survival-related mechanisms. The present study indicated that the DF-A compound possesses activity to enhance anoikis response of the cancer cells by suppressing the level of survival regulatory as well as anti-apoptotic proteins.






Control
Treatment
Relative Mcl-1 level
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
24
Time (h)


Control
Treatment
Relative Bax level
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
24
Time (h)


Control
Treatment
Relative Bcl-2 level
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
6
12
24
Time (h)

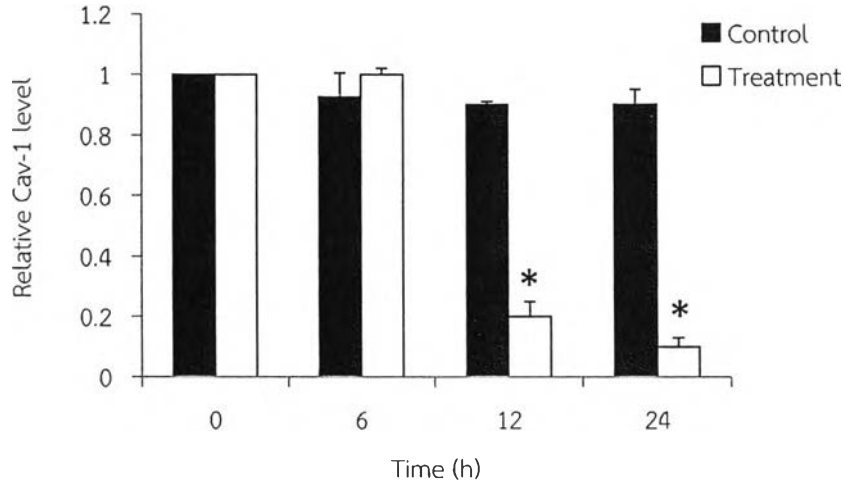


Figure 28 Effects of DF-A on anoikis regulatory proteins. (A) Cells were treated with DF-A (0-5 μM) for indicated times and the levels of activated Akt, total Akt, Mcl-1, Bcl-2, Bax and Cav-1 were determined by western blotting. β-actin was used as loading control to confirm equal loading of the samples. (B) The protein signals were quantified by densitometry and mean data from independent experiments were normalized to the results. Values are means of 3 independent triplicate experiments ± SE. *$p<0.05$ versus untreated control at each indicated time.

5. DF-A inhibits migration of lung cancer cells

As an assessment for cell migration using wound-healing assay may be interfered by the effect of compound in induction or inhibition of cell proliferation. To test whether DF-A has an anti-migration activity, we first determined the possible effect of such a compound on cell proliferation. Human lung cancer cells were cultured in the presence of DF-A for 24-72 h, and cell proliferation was determined by MTT assay. Figure 29 indicated that DF-A at indicated concentrations cause neither

toxic nor proliferative effect on the cells over times. Thus, DF-A at such concentrations were further tested for anti-migration effect.

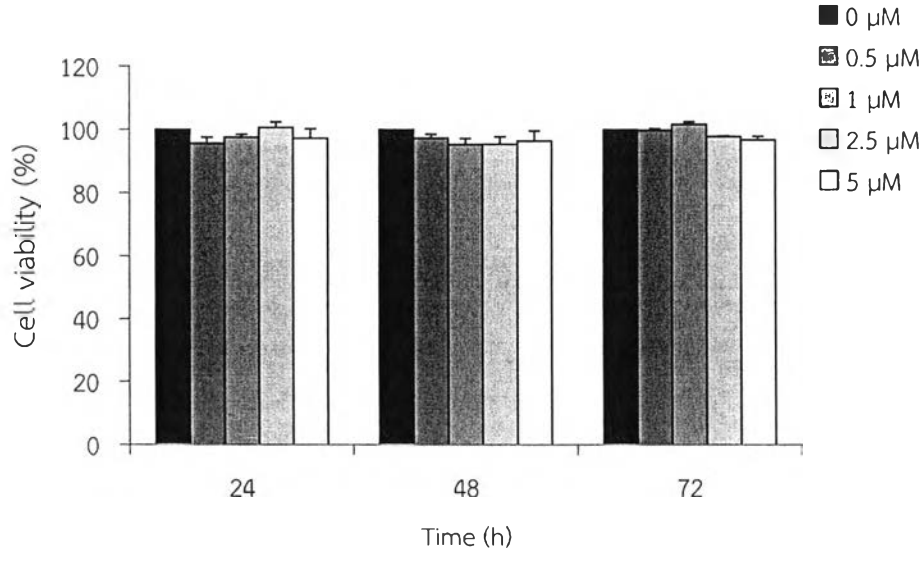
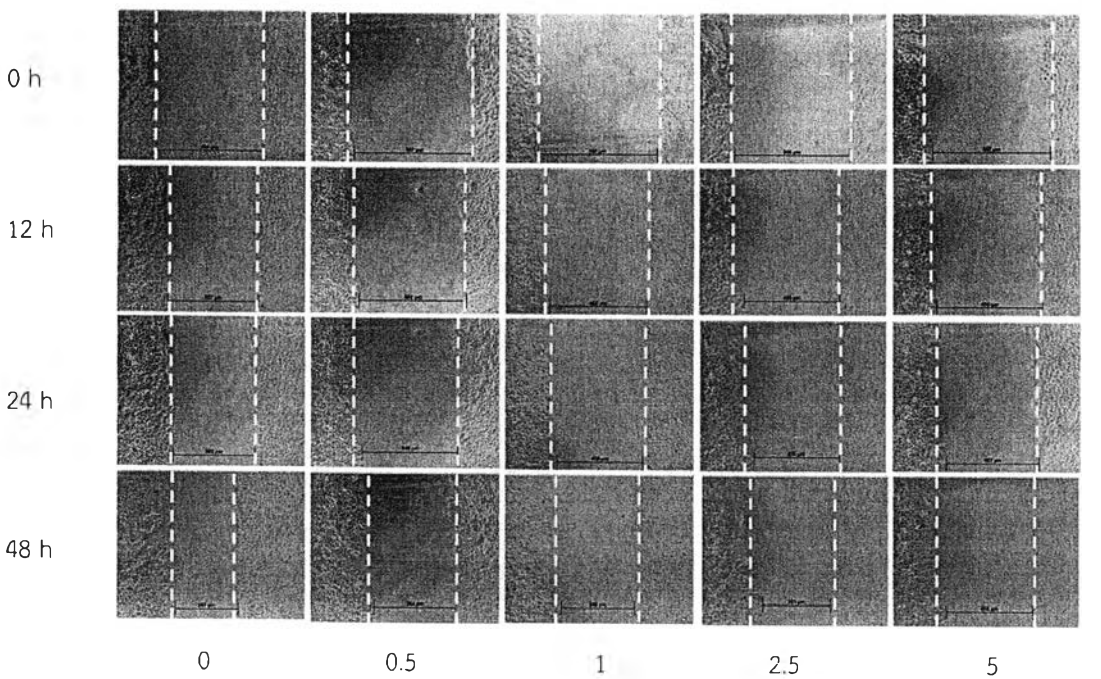


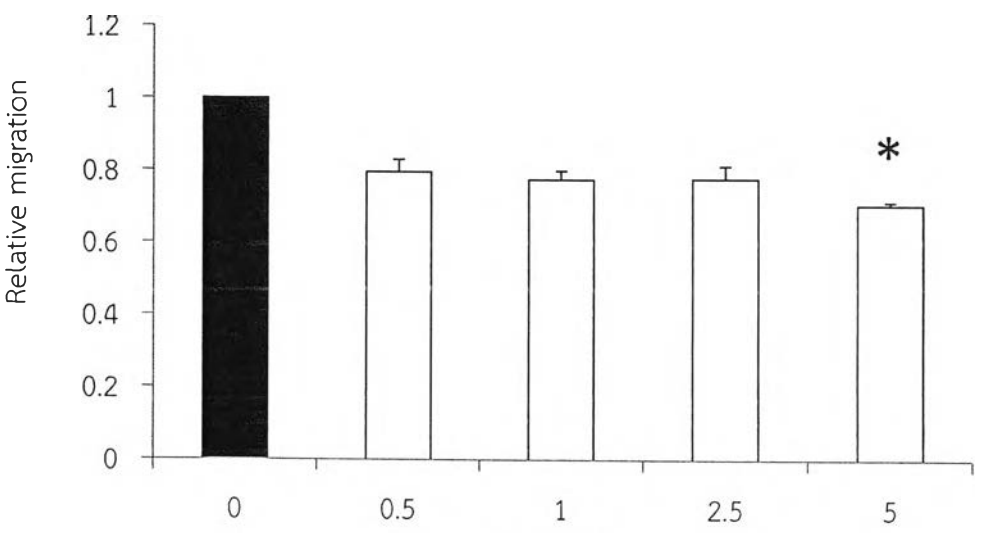
Figure 29 Proliferative effect of DF-A on H460 cells after cells were treated with DF-A (0-5 μM) for 24, 48 or 72 h. The viability of cells was analyzed by MTT assay. Values are means ± SE from 3 independent experiments. *$p < 0.05$ versus untreated control at each indicated time.

A monolayer of cells were scratched and treated with various non-toxic concentrations of DF-A for 12, 24 or 48 h as described in Materials and Methods. Figures 30(B) and 30(C) show that DF-A significantly inhibited migration behavior of the cells, of which approximately 30% inhibition was found in cells treated with 5 μM DF-A compared to that of non-treated control. Also, the inhibitory effect of DF-A on cell migration was observed in a time-dependent manner (Figure 30(C)).

A



B



C

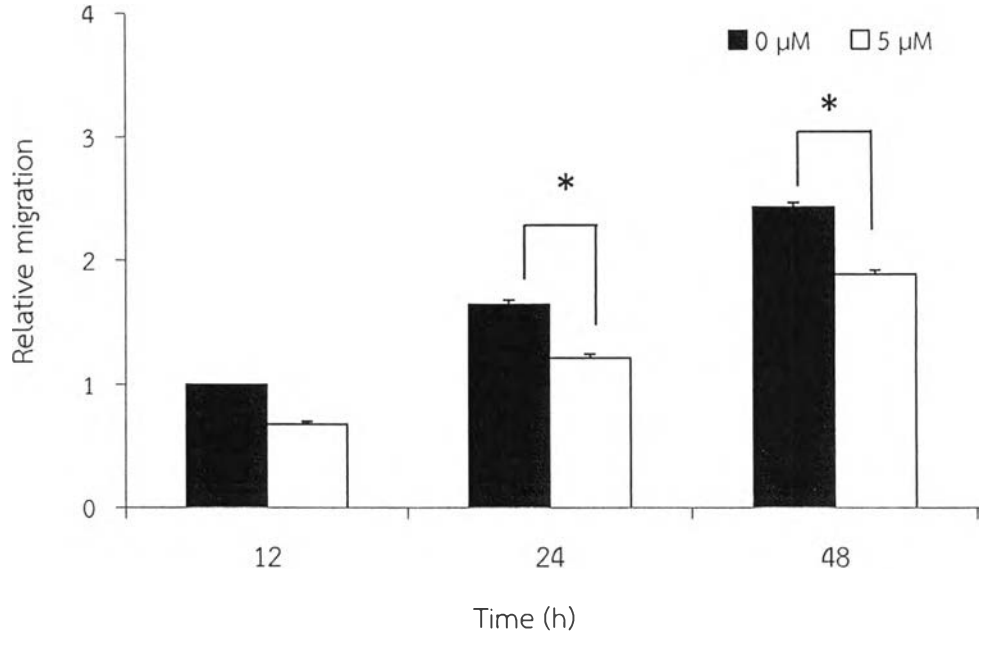
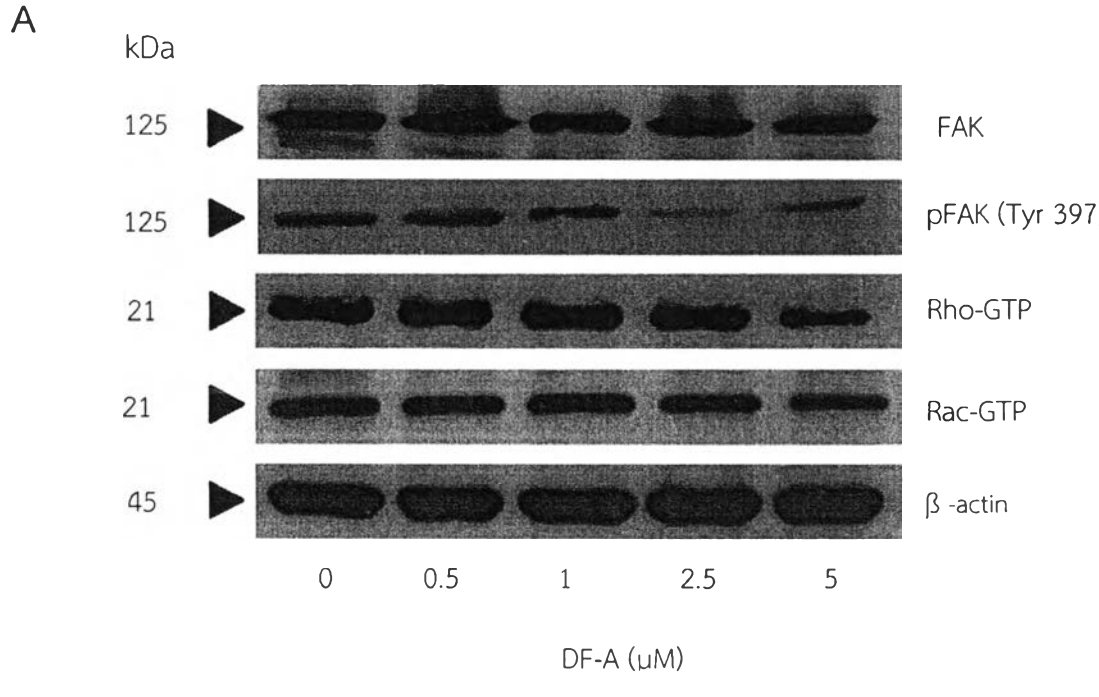


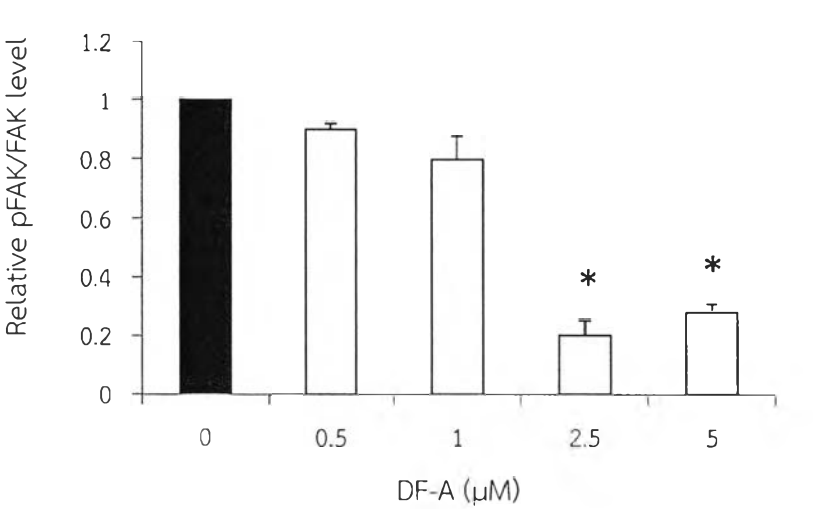
Figure 30 (A) Monolayer of H460 cells were scratched and incubated with DF-A (0-5 μM) for 12, 24 or 48 h. After indicated time, wound space was photographed. (B) After cells were treated with DF-A (0-5 μM) for 24 h, wound space was analyzed and represented as relative migration level by comparing the relative change in wound space of untreated cells. Values are means ± SE from 3 independent experiments. *p < 0.05 versus untreated control. (C) After cells were treated with DF-A (5 μM) for 12, 24 and 48 h, wound space was analyzed and represented as relative migration level by comparing the relative change in wound space of untreated cells. Values are means ± SE from 3 independent experiments. *p < 0.05 versus untreated control.

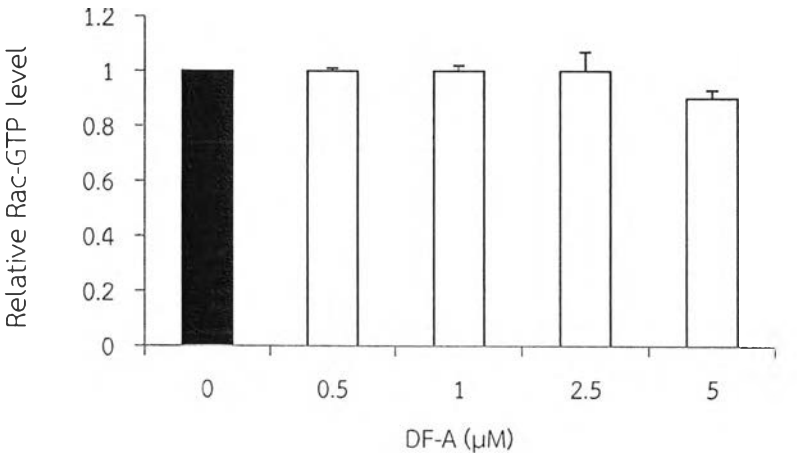
6. DF-A decreases cell migration via suppression of FAK and Rho activations.

Having shown that DF-A possess anti-migration effect, we further investigated the underlying mechanism. Cells were incubated DF-A and allowed to migrate for 24 h and the levels of proteins involving in cell migration, namely pFAK, Rho-GTP, and Rac-GTP, were determined by western blotting. Figure 31 shows that DF-A at the concentrations of 2.5 and 5 μM significantly decreased the level of activated FAK (pFAK (Tyr 397)). In addition, the active forms of Rho (Rho-GTP) was found to be decreased in the cells treated with DF-A. However, the active Rac (Rac-GTP) was only slightly altered in the DF-A treated cells. Together, these results indicate that DF-A inhibits migration of H460 cells by suppressing the activation of FAK and Rho.



B


Relative pFAK/FAK level
1.2
1
0.8
0.6
0.4
0.2
0
*
*
0
0.5
1
2.5
5
DF-A (μM)


Relative Rac-GTP level
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
1
2.5
5
DF-A (μM)

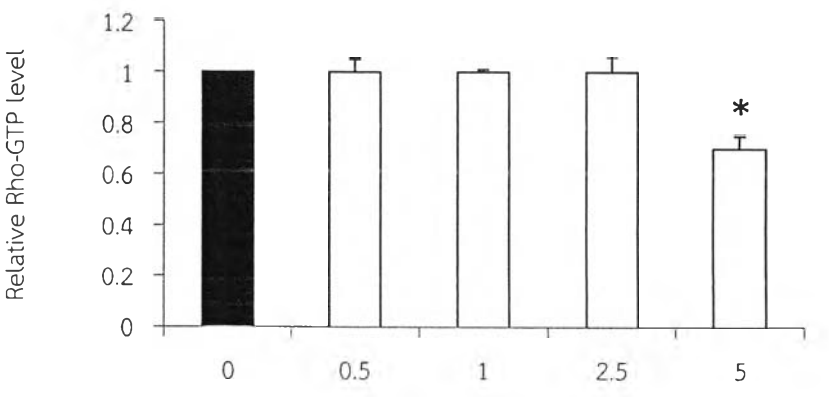

Relative Rho-GTP level
1.2
1
0.8
0.6
0.4
0.2
0
*
0
0.5
1
2.5
5

Figure 31 DF-A suppresses the expression of pFAK and Rho-GTP in H460 cells. (A) H460 cells were treated with DF-A (0-5 μM) for 24 h and the level of migratory-related proteins were determined by western blotting. Cells were collected and analyzed for FAK protein, pFAK (Tyr 397), active Rho-GTP and active Rac-GTP. β-actin was used as loading control to confirm equal loading of the samples. (B) The protein signals were quantified by densitometry and mean data from independent experiments were normalized to the results. Values are means ± SE from 3 independent experiments. *$p<0.05$ versus untreated control.

7. DF-A exerts no significant effects on susceptibility of cancer cells to chemotherapeutic agents

As drug sensitizing activity of compound may benefit the therapeutic outcome of cancer treatment, we tested whether DF-A could sensitize the cells to cisplatin and etoposide-mediated death. Figure 32 shows that the cells treated with 100 μM cisplatin or 100 μM etoposide alone for 24 h, showed approximately 47.23% and 73.46% viability, respectively. Unfortunately, an addition of DF-A to the cells treated with cisplatin or etoposide did not affect the obtained cell viability, suggesting that DF-A at indicated concentrations exerts no drug sensitizing effect.

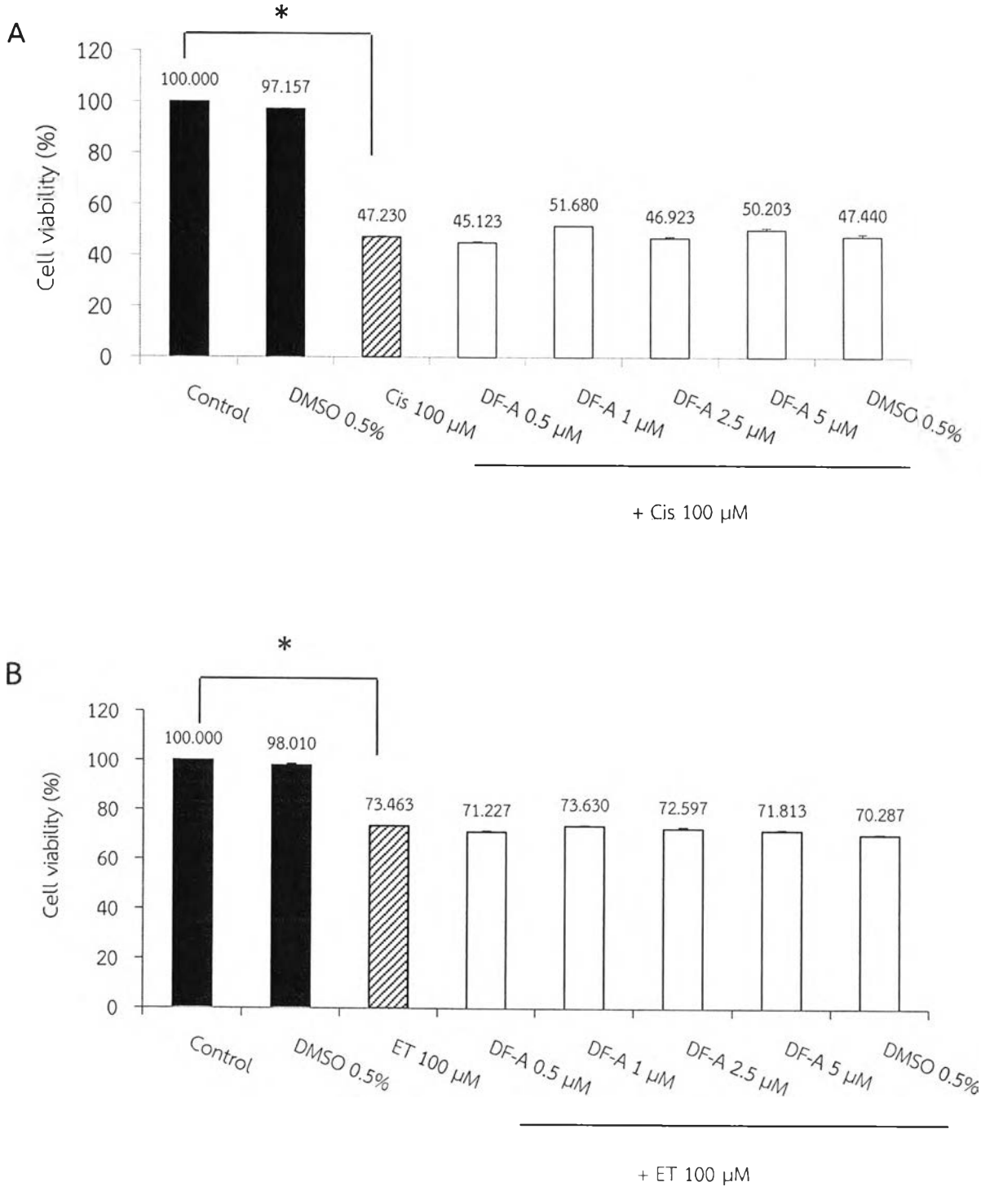


Figure 32 Effect of DF-A on anti-cancer drugs-induced cell death. H460 cells were pre-treated with various non-toxic concentration of DF-A (0-5 µM) for 24 h, prior to

incubated with either 100 μM cisplatin (Cis; (A)) or 100 μM etoposide (ET; (B). The percentage of cell viability was determined by MTT assay. Values are means ± SE from 3 independent experiments. *$p < 0.05$ versus either cisplatin- or etoposide-treated cells.