CHAPTER IV

RESULTS

1. Investigation of cytotoxic effect of TCS on H460 cells

In this study, to investigate the cytotoxic effect of TCS on H460 cells, cells were incubated with TCS at the concentrations of 0-10 μM for 24 h and cell viability was assessed by MTT assay. The result showed that TCS treatment significantly decreased cell survival at the dose of 10 μM with approximately 80% of cells remaining viable, while treatment of the cells with TCS at 0-7.5 μM caused no significant toxic effect (Figure 29).

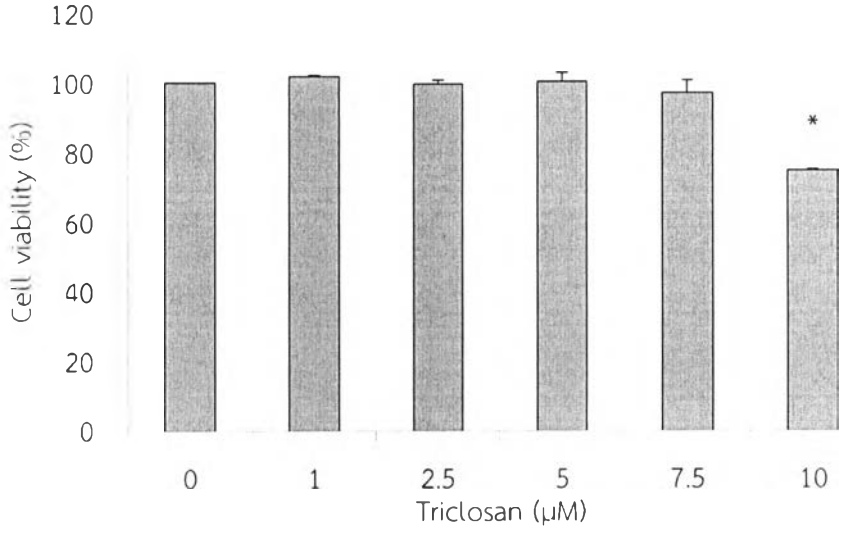


Figure 29 The cytotoxic effect of triclosan (TCS) on H460 cells. After treatment with TCS (0-10 μM) for 24 h, percentage of cell viability was determined by MTT assay. Values are means of three independent triplicate samples ± SEM. *$P < 0.05$ versus non-treated control.

2. Investigation on mode of cell death induced by TCS in H460 cells

Mode of cell death after TCS treatment was investigated by Hoechst33342/PI staining assay. The results confirmed that apoptosis and necrosis were not detected in the TCS-treated cells at 0-7.5 μM. The apoptotic cells with fragmented or condensed nuclei were only detected in the cells treated with 10 μM TCS (Figure 30). Therefore, the concentrations of TCS at 0-7.5 μM were used for the subsequent experiments.

3. Investigation the effect of TCS on anoikis of H460 cells

To determine the effect of TCS on cell anoikis, H460 cells were detached and suspended in culture media containing TCS (0–7.5 μM) in ultra-low attachment 6-well plate for 0-24 h. Cell survival was determined by MTT assay. As shown in Figure 31, TCS-treated cells showed no significant difference in terms of cell anoikis in comparison to that of the non-treated control cells at each time points.

4. Investigation of cell viability in response to TCS on AR cells

AR cells were generated as described in Materials and Methods. Detached H460 cells spontaneously formed multicellular aggregates after culture for 48 h (Figure 32).

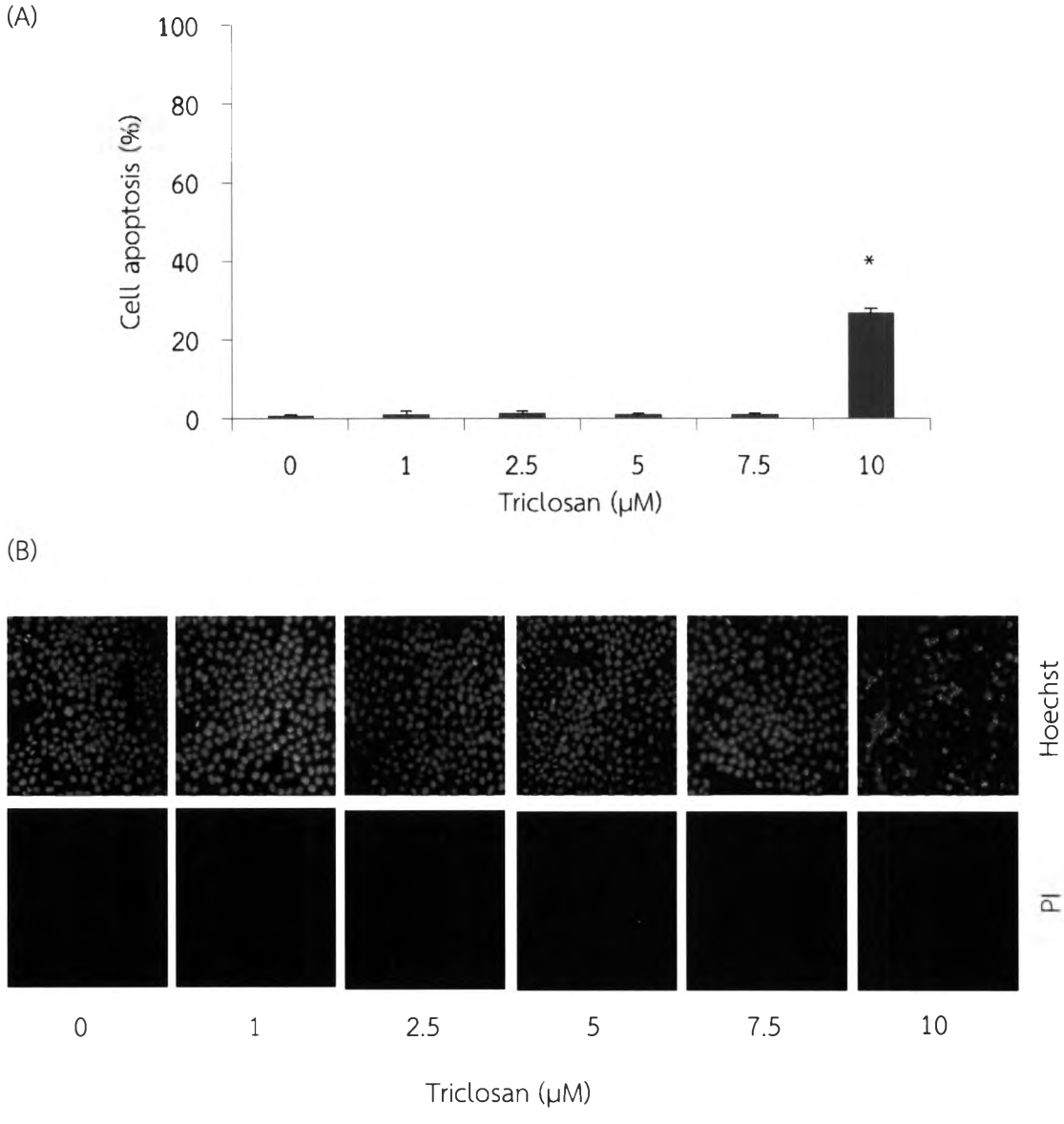


Figure 30 Mode of cell death induced by triclosan (TCS). After treatment with TCS (0-10 μM) for 24 h, the percentage of apoptotic cells were detected by Hoechst33342 staining (A). Values are means of three independent triplicate samples ± SEM. *P < 0.05 versus non-treated control. After indicated treatment, nuclear morphology of the cells were detected by Hoechst33342/PI co-staining assay and visualized under a fluorescence microscope (B).

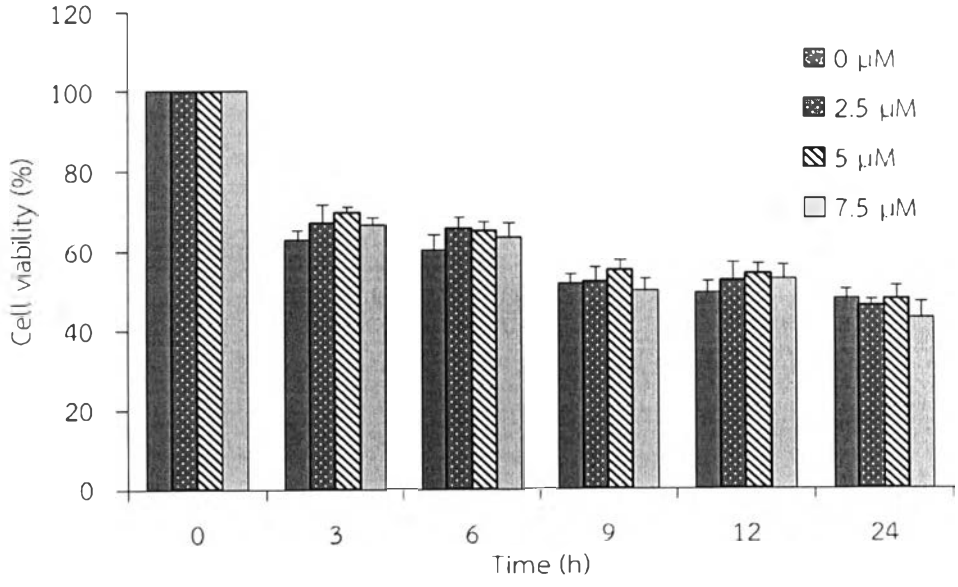


Figure 31 The effect of triclosan (TCS) on H460 cells anoikis. Cells were treated with TCS (0-7.5 μM) for 0, 3, 6, 9, 12, and 24 h in detached condition. Cell survival was determined by MTT assay at indicated time. Data represent the means of three independent triplicate samples ± SEM.

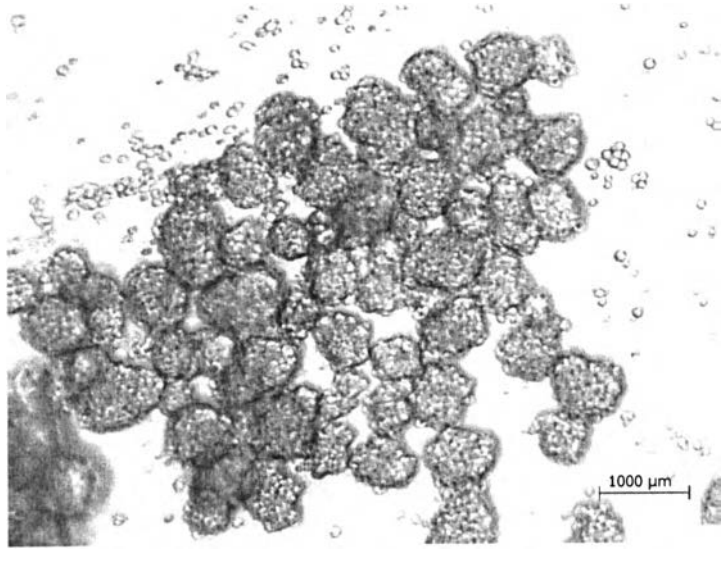


Figure 32 Multicellular aggregation of anoikis resistant cells (AR cells). Scale bar is 1,000 μm.

To confirm the non-cytotoxic effect of TCS on AR cells, cell viability of AR cells in response to TCS was investigated. AR cells were incubated with TCS at the concentrations of 0-7.5 μM for 12, 24 and 48 h in detached condition and cell viability was assessed by PrestoBlue assay. The result showed that treatment of AR cells with TCS at these concentrations caused no significant toxic effect to cells in detached condition (Figure 33).

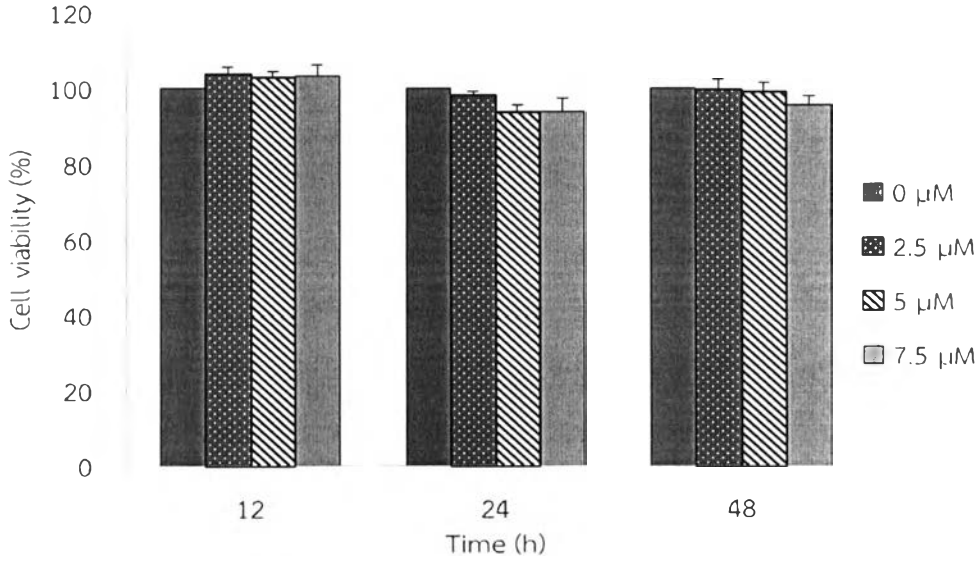


Figure 33 The effect of triclosan (TCS) on anoikis resistant cell (AR cell) viability in detached condition. After treatment with TCS (0-7.5 μM) for 12, 24 and 48 h, percentage of cell viability was determined by PrestoBlue assay. Values are means of three independent triplicate samples ± SEM.

5. Investigation the effect of TCS on cell-cell interaction of AR cells

To examine the effect of TCS on cell-cell adhesion of AR cells, cells were seeded in 24-well low attach plate at the density of 1.5×10^5 cells/well and treated with non-toxic concentrations of TCS. Cell-cell interaction as observed by the formation of cell aggregation which was photographed using a phase-contrast microscope. Aggregate size and number was determined and calculated relative to

the non-treated control. The result showed that TCS treatment significantly altered the aggregate behavior of the cells to single cell suspension (Figure 34). Addition of TCS resulted in the significant reduction of both number and size of multicellular aggregates in a concentration-dependent manner (Figure 35).

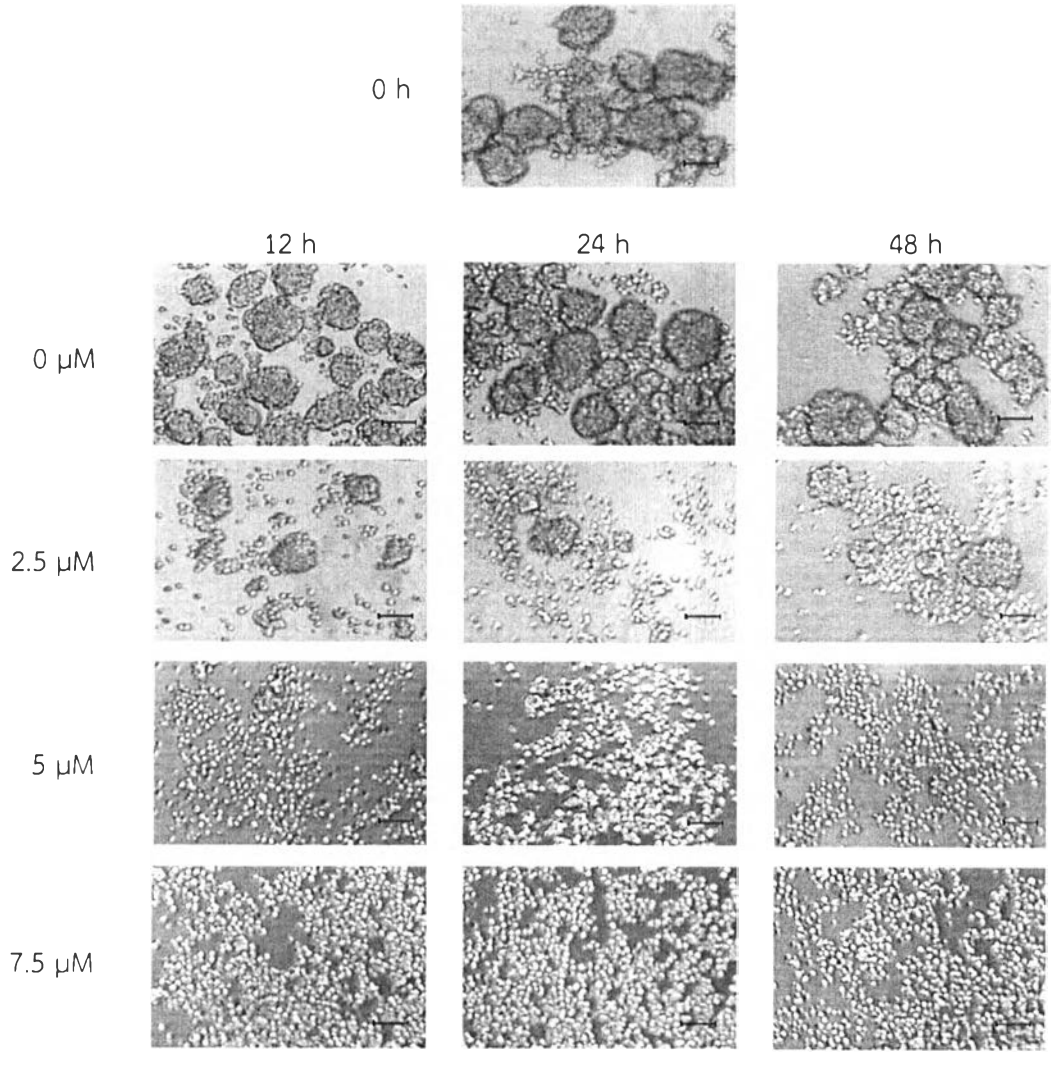


Figure 34 The effect of triclosan (TCS) on cell-cell interaction of anoikis resistant cells (AR cells). Cells were treated with TCS (0-7.5 μM) for 12, 24 or 48 h in detached condition and cell-cell interaction was photographed. Scale bar is 1,000 μm.

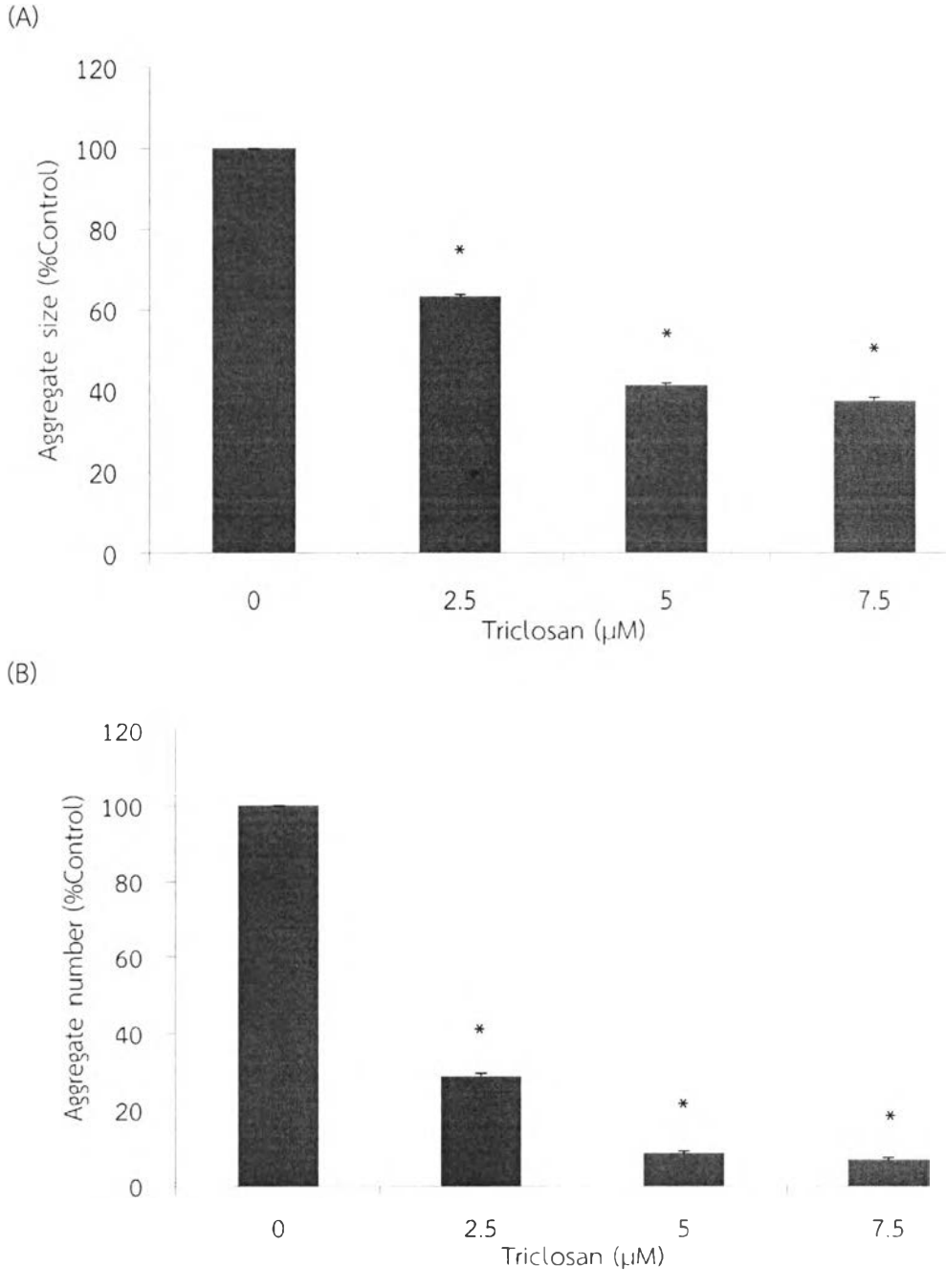


Figure 35 Aggregate size and aggregate number of anoikis resistant cells (AR cells) after treatment with triclosan (TCS) at 0-7.5 μM for 24 h in detached condition. Aggregate size (A) and aggregate number (B) were determined by image analyzer. Data present means of three independent triplicate samples ± SEM. *$P < 0.05$ versus non-treated control.

6. Investigation the molecular mechanisms of EMT inducing effect of TCS on AR cells

Effect of TCS treatment on the expression of E-cadherin and EMT markers including N-cadherin, vimentin, snail, and slug were investigated. AR cells were treated with non-toxic concentrations of TCS for 24 h and EMT markers were evaluated by western blotting. Figure 36 and 37 show that the expression of E-cadherin was significantly decreased in response to TCS treatment in a concentration-dependent manner. In addition, TCS significantly enhanced the increase the expression of N-cadherin, vimentin, slug, and snail. The expression of snail was significantly enhanced by TCS treatment at 5 and 7.5 μM in a concentration-dependent manner, while the expression of slug was only significantly induced by the treatment with TCS at 7.5 μM.

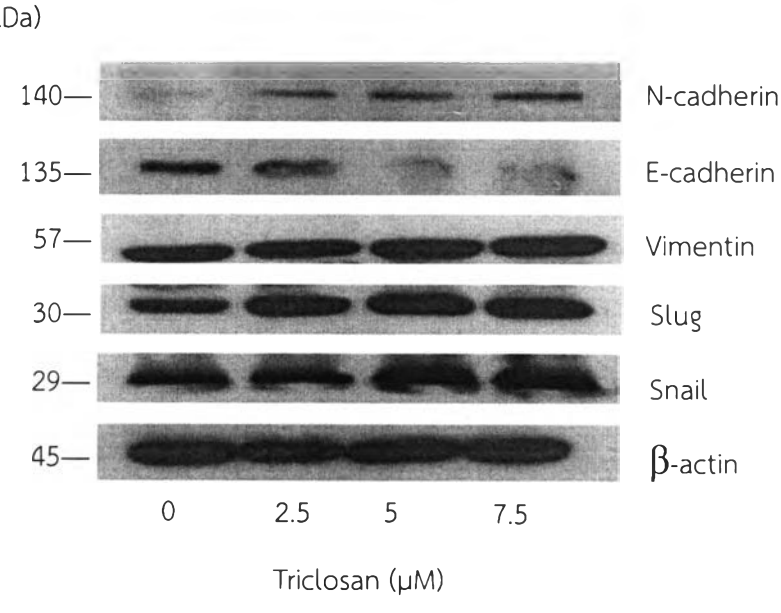
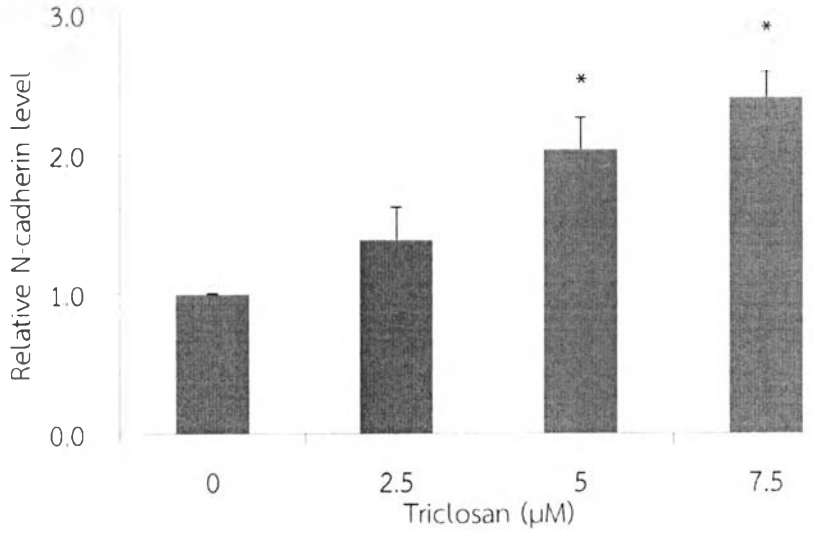


Figure 36 Effect of triclosan (TCS) on E-cadherin and EMT markers. Anoikis resistant cells (AR cells) were treated with TCS (0-7.5 μM) for 24 h in detached condition. The level of N-cadherin, E-cadherin, vimentin, slug and snail were determined by western blotting. Blots were reprobed with β-actin to confirm equal loading.

(A)



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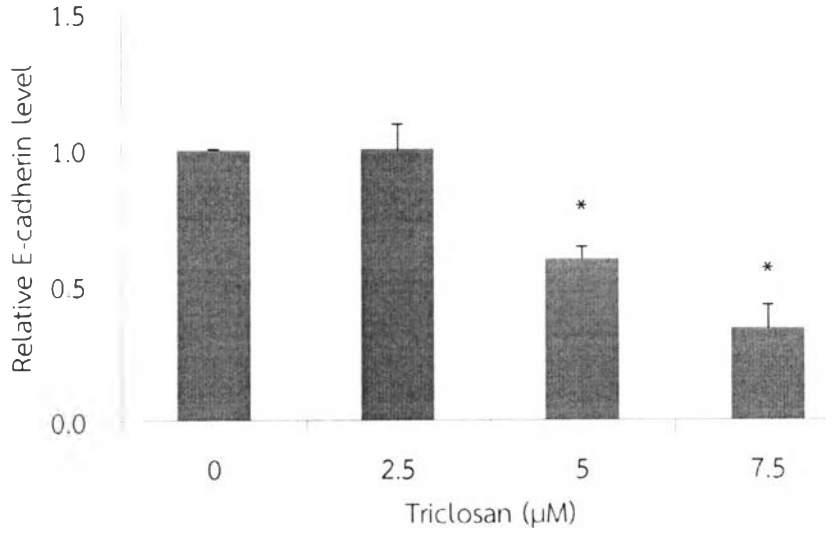

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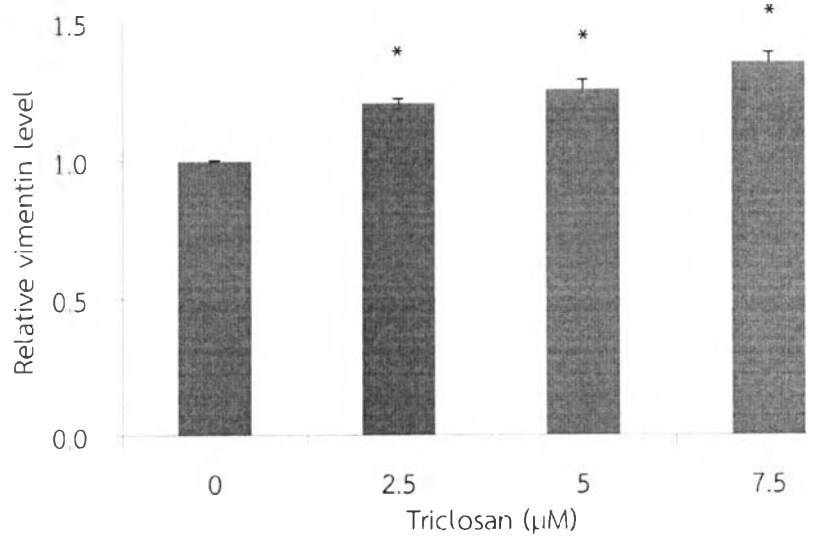


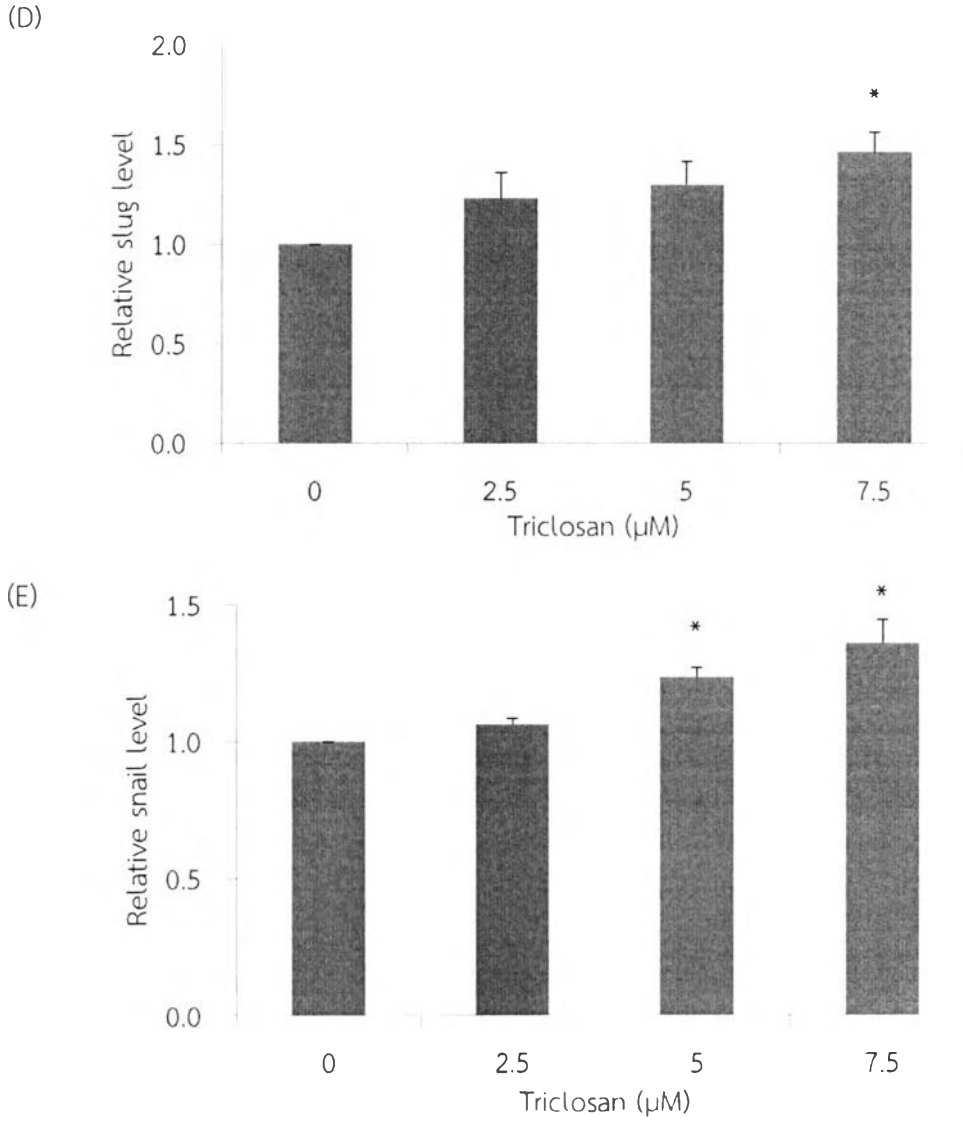


Figure 37 The immunoblot signals of EMT-related proteins quantified from Figure 36. The immunoblot signals of N-cadherin (A), E-cadherin (B), vimentin (C), slug (D) and snail (E) were quantified by densitometry and mean data from independent experiments were normalized to the results. Data present means of three independent triplicate samples ± SEM. *P < 0.05 versus non-treated control.

7. Investigation the effect of TCS-mediated EMT on tumorigenic ability of AR cells

To investigate the tumorigenic ability or colony-forming ability of TCS-treated AR cells in anchorage-independent condition using colony formation assay. AR cells were treated with TCS for 24 h before subjected to the assay. Cells were then seeded in agarose layer to prevent cell-cell interaction and attachment. Colonies number and colony size were obtained by photographing and counting after the cells were cultured for 7 and 10 days. The colony formation was shown in Figure 38. Colony number and colony size of each treatment were calculated as a percent of control group and shown in Figure 39A and 39B, respectively. The results indicated that TCS at the concentrations of 5 and 7.5 μM significantly increased colony formation of AR cells. However, TCS at both concentrations significantly reduced colony size in comparison to that of the non-treated control group. Such observations indicated that TCS-mediated EMT promoted tumorigenic ability of the AR cells, but decreased the growth rate of the cells in detached condition.

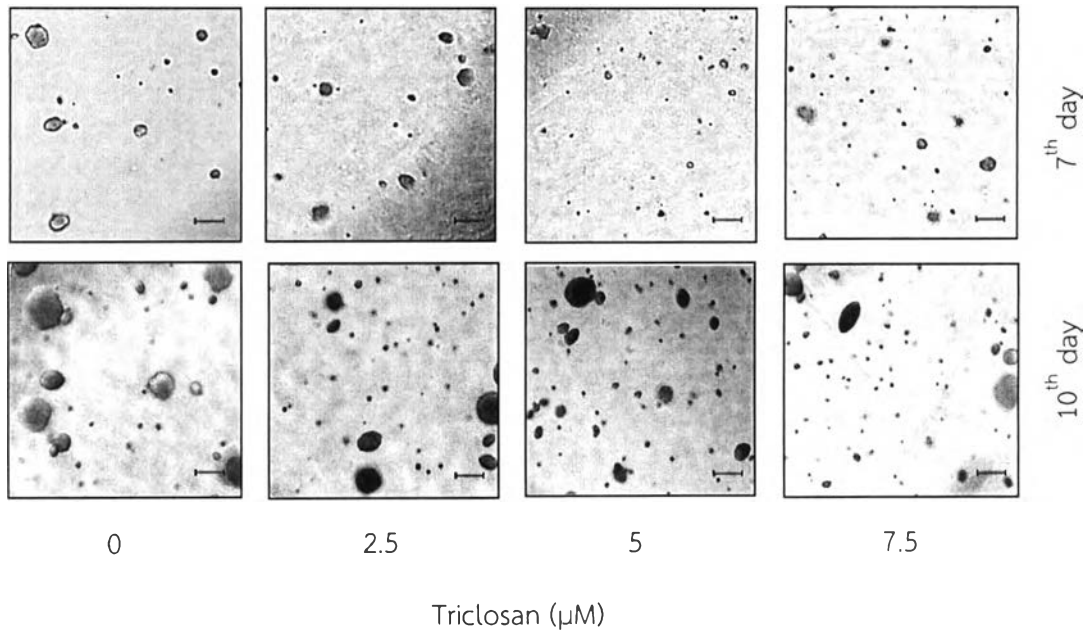


Figure 38 Effect of TCS-mediated EMT on tumorigenic ability of anoikis resistant cells (AR cells). Cells were pretreated with TCS (0-7.5 μM) for 24 h and subjected to soft agar colony formation assay, as described in ''Materials and Methods''. Representative fields from three independent experiments were photographed after the cells were cultured for 7 and 10 days. Scale bar is 1,000 μm.

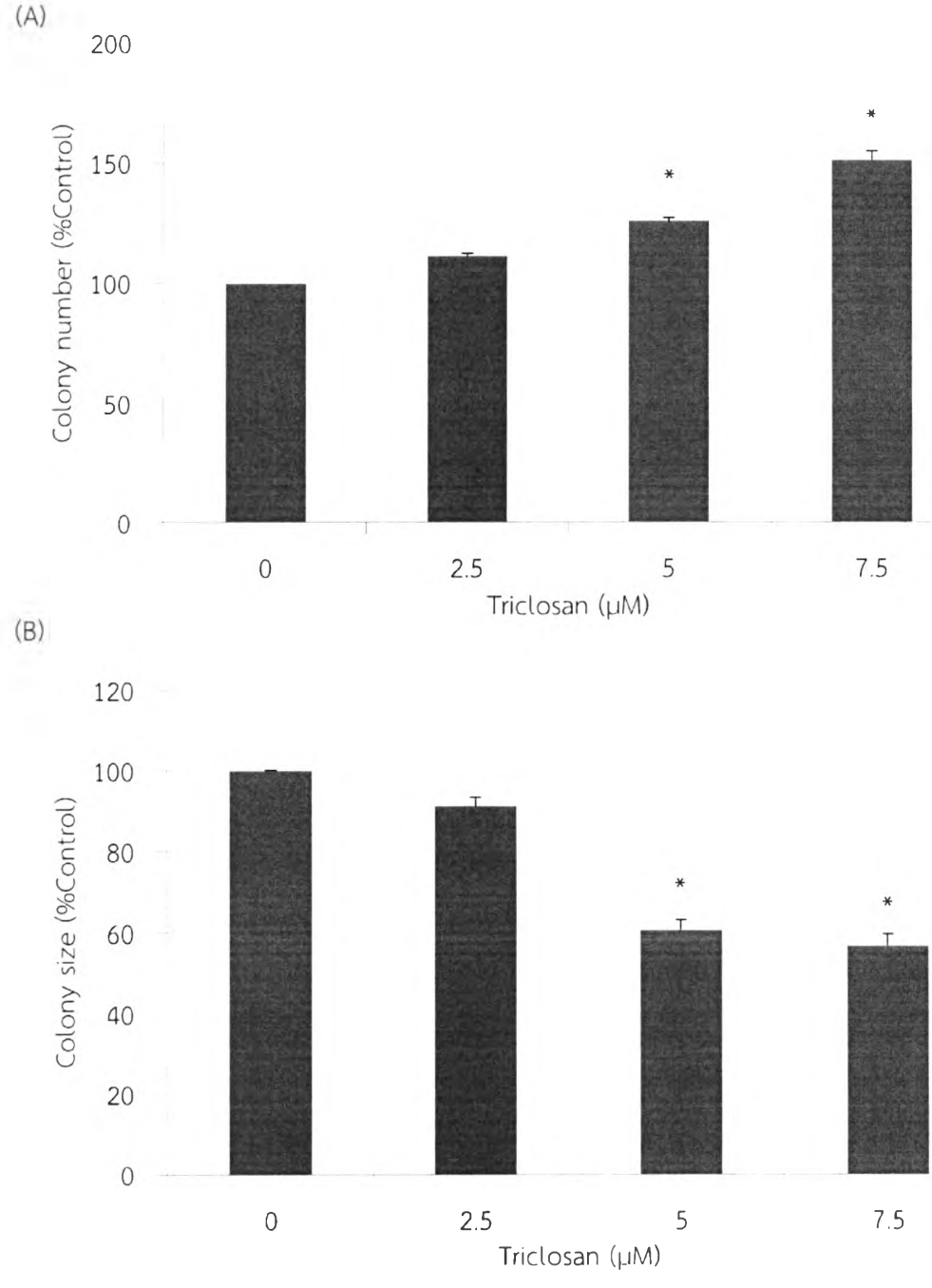


Figure 39 Colony number and colony size in response to triclosan (TCS) of anoikis resistant cells (AR cells). Colony number (A) and colony size (B) were determined by image analyzer on 10^{th} day of culture. Values are means of three independent triplicate samples ± SEM. *$P < 0.05$ versus non-treated control.

8. Investigation the effect of TCS-mediated EMT on cell polarity and filopodia formation of AR cells in attachment condition

To study the effect of TCS-mediated EMT on cell polarity and filopodia formation, AR cells were treated with TCS at non-toxic concentrations for 24 h and the migration and invasion behaviors of the cells were determined as described in Materials and Methods. Cell polarity and filopodia stained with phalloidin-rhodamine were shown in Figure 40A and Figure 40B, respectively. The results showed that TCS-treated cells exhibited increased polarity and filopodia. TCS-treated cells exhibited filopodia protrusions accumulating at the border of cells in a concentration-dependent fashion. These results suggested that EMT in response to TCS treatment induced cell polarity and filopodia formation of AR cells.

9. Investigation the effect of TCS-mediated EMT on AR cell migration

To investigate AR cell migration after treatment with TCS, the migratory ability of cells was evaluated using Boyden chamber assay. Migration assay revealed that TCS at 5 and 7.5 μM significantly increased cell migration (Figures 41). These results suggested that TCS-mediated EMT potentiated migratory ability of AR cells.

10. Investigation the effect of TCS-mediated EMT on AR cell invasion

To investigate AR cell invasion after treatment with TCS, the invasion behavior of cells was evaluated using Boyden chamber assay with matrigel-coated transwells on the upper surface of chamber. The results revealed that TCS at 5 and 7.5 μM significantly increased cell invasion (Figures 42). These results suggested that TCS-mediated EMT potentiated invasive ability of AR cells.

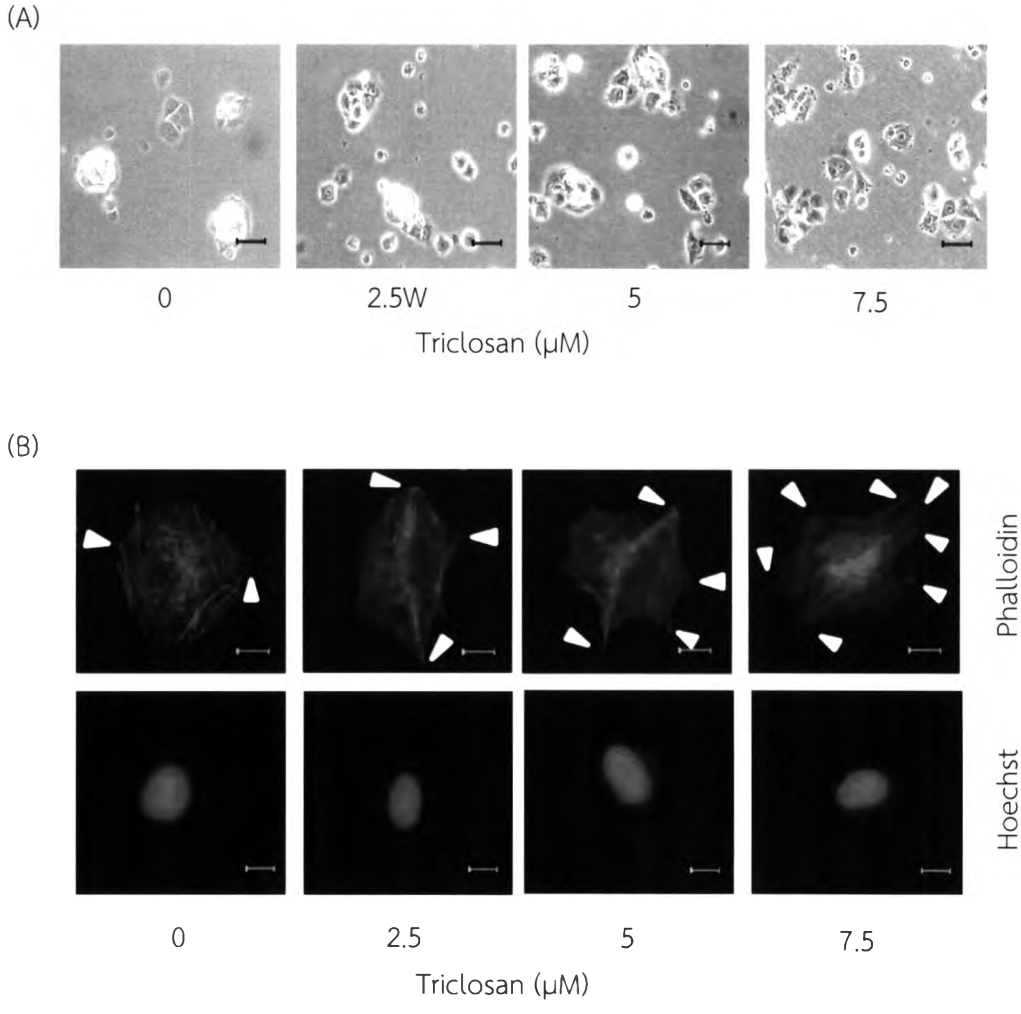


Figure 40 Effects of TCS-mediated EMT on cell polarity and filopodia formation of anoikis resistant cells (AR cells). Cells were treated with TCS (0-7.5 μM) for 24 h and then attach on conventional culture dishes for 4 h. Cell morphology was detected by phase contrast microscopy. Scale bar is 50 μm (A). After indicated treatment, filopodia and viable cells were detected by phalloidin-rhodamine or Hoechst33342 staining, respectively (B). Cells were visualized under fluorescence microscope. Filopodia protrusions of each treatment were indicated by arrows. Scale bar is 5 μm.

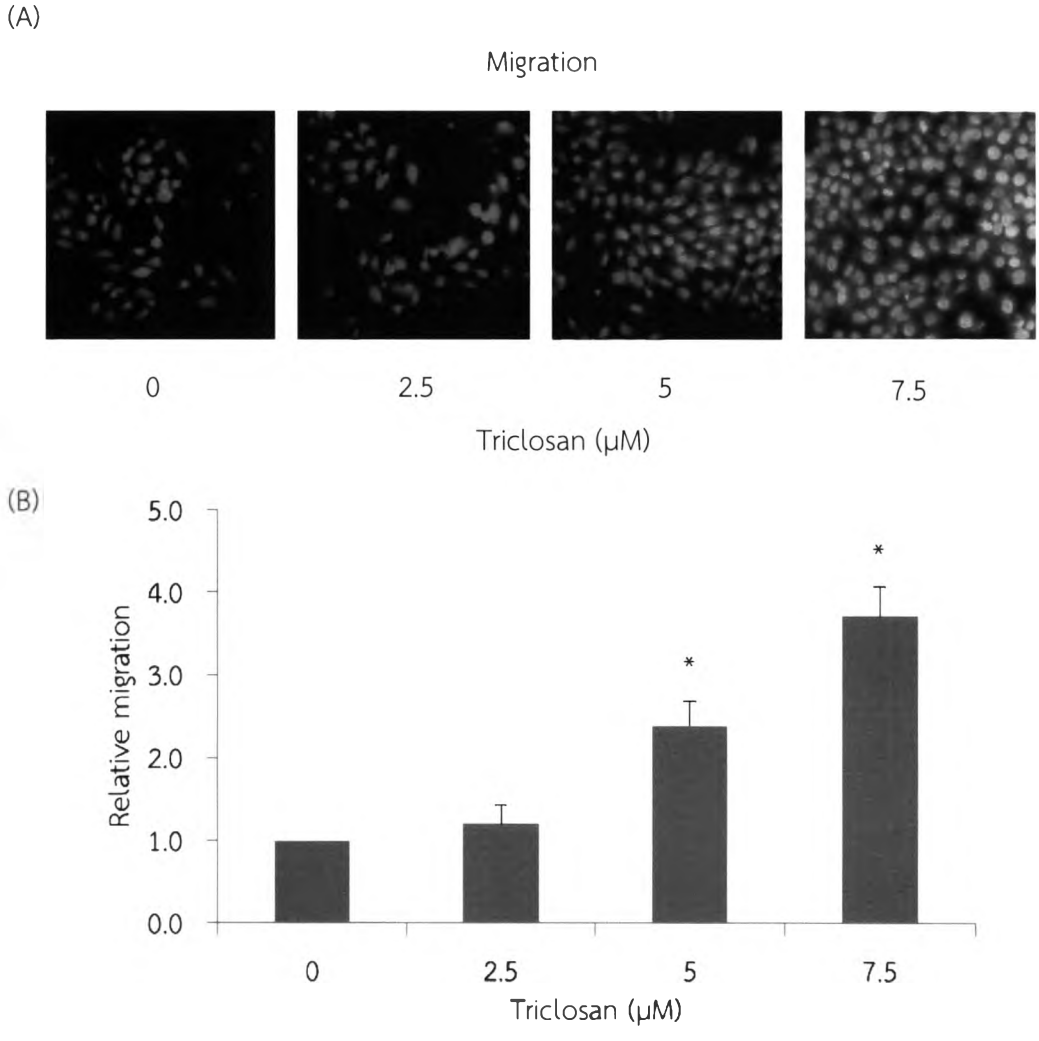


Figure 41 Effects of TCS-mediated EMT on migratory ability of anoikis resistant cells (AR cells). Cells were pretreated with TCS (0-7.5 μM) for 24 h. Transwell assay was used to investigate cell migration. Migratory cells at the basolateral side of membrane were stained with Hoechst33342 and visualized under fluorescence microscopy (A). An average number of migratory cells in each field at the basolateral side of membrane were plotted relative to control group (B). Values are means of three independent triplicate samples ± SEM. *$P < 0.05$ versus non-treated control.

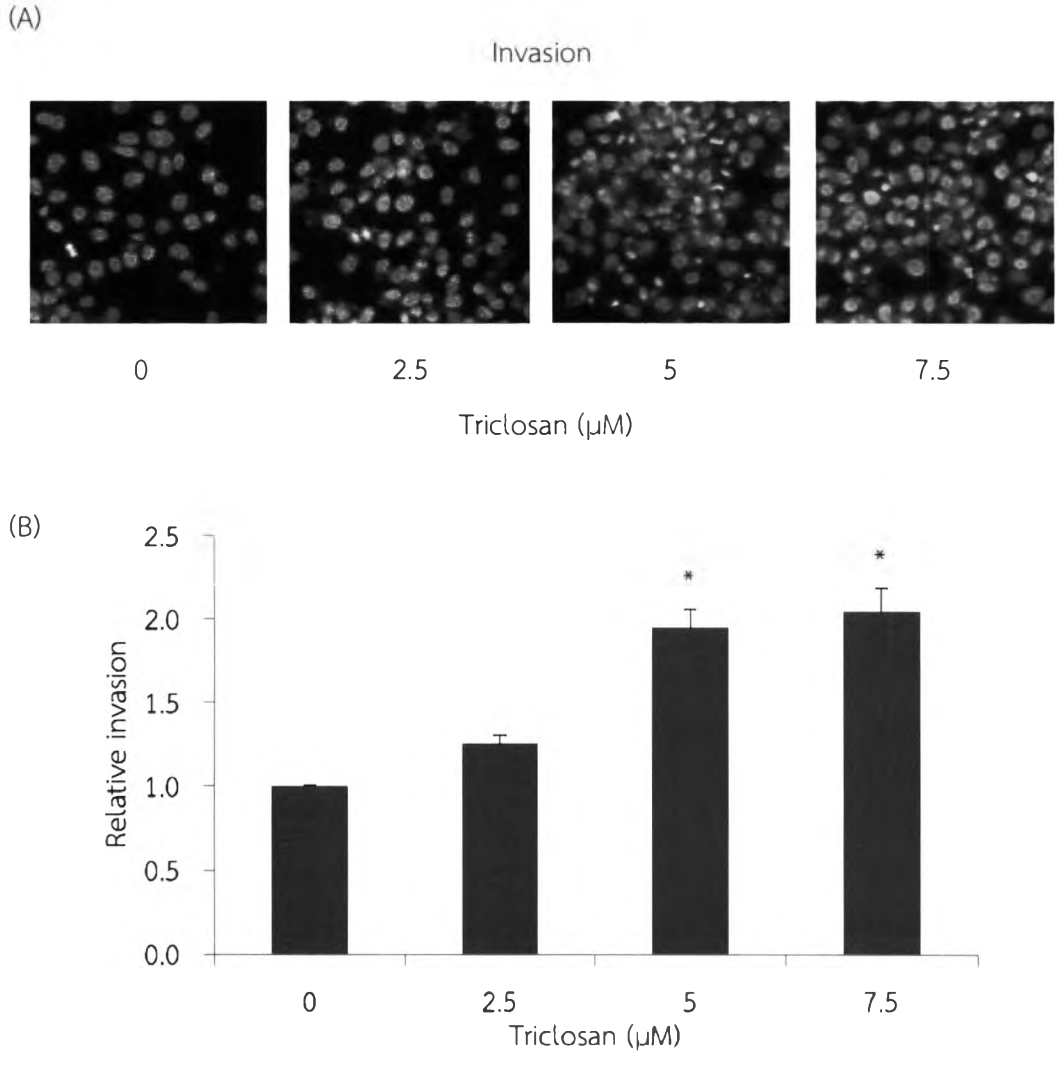


Figure 42 Effects of TCS-mediated EMT on invasive ability of anoikis resistant cells (AR cells). After treatment with TCS (0-7.5 μM) for 24 h, cell invasion was evaluated using transwell coated with matrigel as described in ''Materials and Methods''. Invaded cells at the basolateral side of membrane were stained with Hoechst33342 and visualized under fluorescence microscopy (A). An average number of invaded cells in each field across the membrane were plotted relative to control group (B). Values are means of three independent triplicate samples ± SEM. *$P < 0.05$ versus non-treated control.

11. Investigation the molecular mechanisms of TCS-mediated EMT on AR cell motility

Further, the down-stream effector proteins which are responsible for cell motility were determined using western blotting. The cells were treated with the indicated concentrations of TCS for 24 h and subjected to western blot analysis. The expression levels of migratory-related proteins including activated FAK (phosphorylated FAK, Tyr 397), FAK, activated Akt (phosphorylated Akt, Ser 473), Akt, activated Rac1 (Rac1-GTP), and activated RhoA (RhoA-GTP) were investigated. As shown in Figure 43 and 44, TCS treatment significantly increased the level of phosphorylated FAK, activated Akt, and active Rac1-GTP. However, TCS possessed no significant effect on activated RhoA level. These results suggested that TCS-induced EMT promoted the motility of AR cells through the activation of FAK/Akt signaling pathway as well as Rac1 activation.

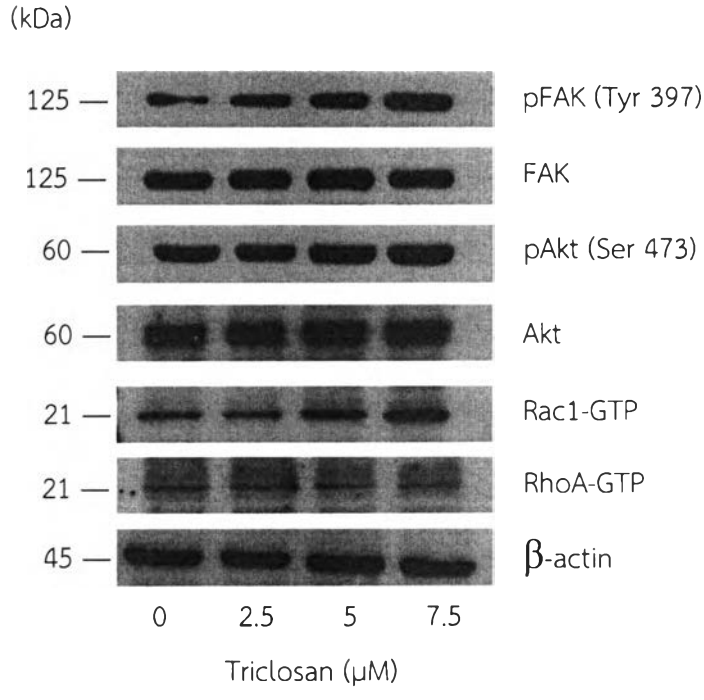
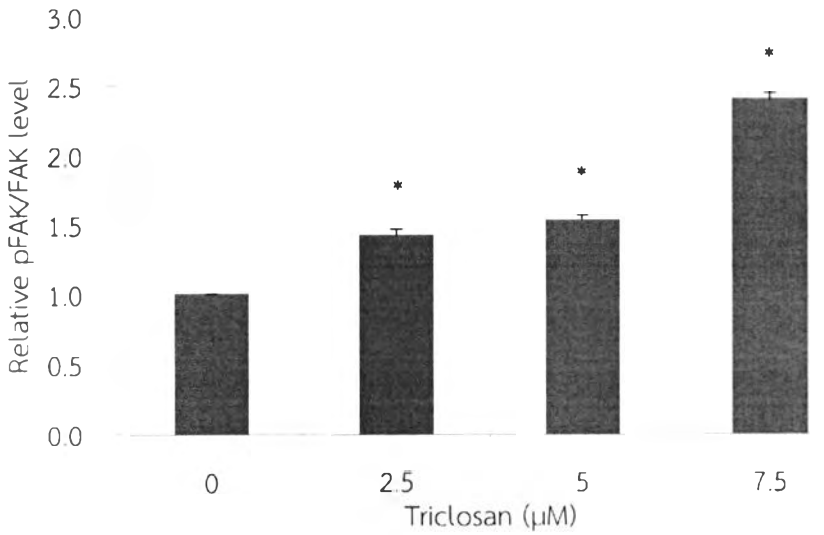


Figure 43 Effect of TCS-mediated EMT on migratory-related proteins. Anoikis resistant cells (AR cells) were treated with TCS (0-7.5 μM) for 24 h in detached condition and then attach on conventional culture dishes for 4 h. The level of pFAK (Tyr 397), FAK, pAkt (Ser 473), Akt, activated Rac1 (Rac1-GTP) and activated RhoA (RhoA-GTP) were determined by western blotting. Blots were reprobed with β-actin to confirm equal loading.

(A)



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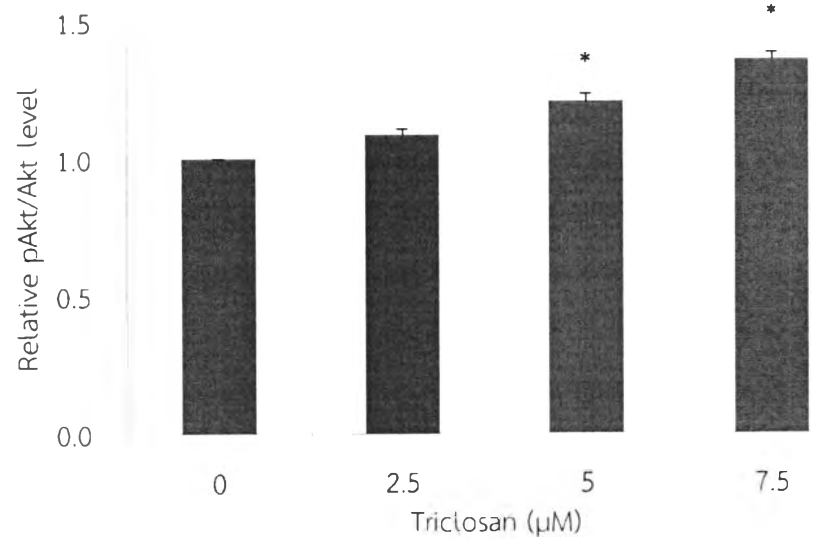

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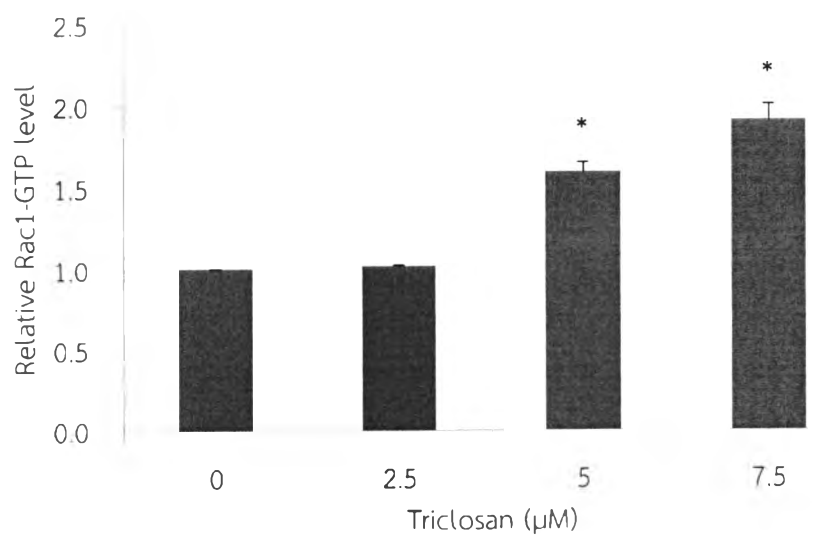

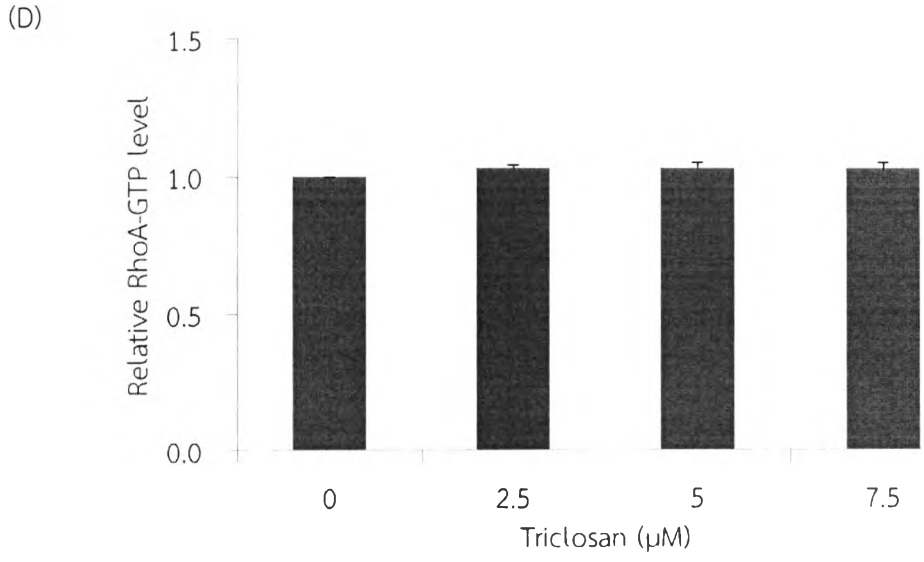

Figure 44 The immunoblot signals of migratory-related proteins quantified from Figure 43. The immunoblot signals of pFAK/FAK (A), pAkt/Akt (B), Rac1-GTP (C) and RhoA-GTP (D) were quantified by densitometry and mean data from independent experiments were normalized to the results. Values are means of three triplicate independent samples ± SEM. *P < 0.05 versus non-treated control.