

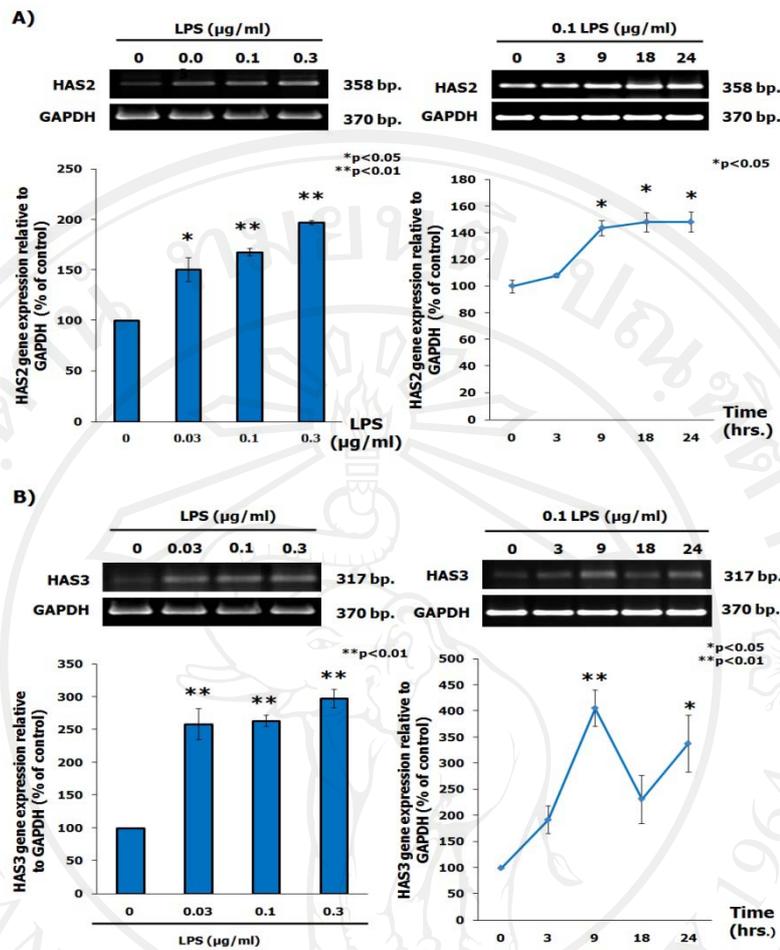
## CHAPTER III

### RESULTS

#### 3.1 Effects of Lipopolysaccharide (LPS) on human synovial fibroblast cells.

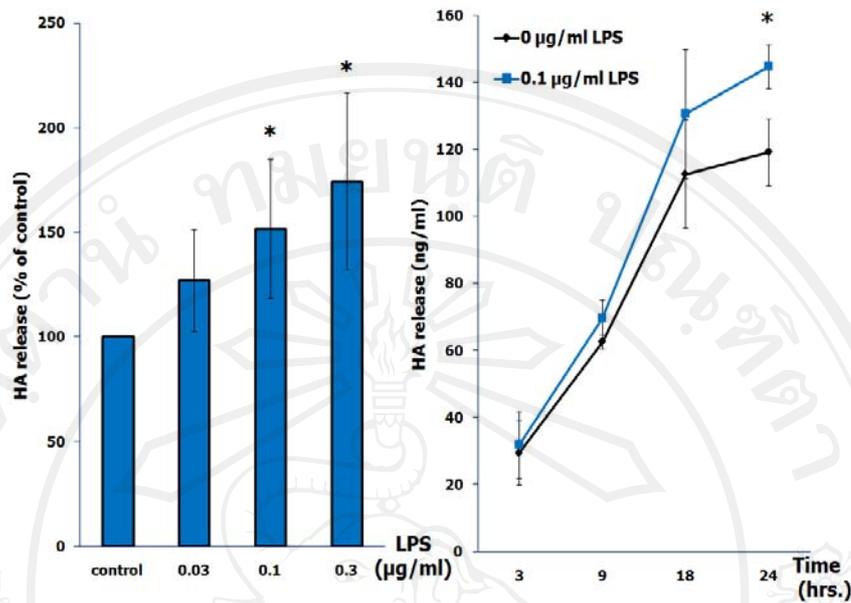
In order to find optimal dose and time of LPS stimulation, LPS (0.03-0.3  $\mu\text{g/ml}$ ) was used to induce human synovial fibroblast cells for 0-24 hours. Next, cells were harvested and media were collected. Total RNA from lysed-cell were extracted and determined the expressions of HAS2 and HAS3 genes using RT-PCR. The collected culture media analyzed the level of hyaluronan using ELISA technique A.

It was found that exposure of 0.1  $\mu\text{g/ml}$  LPS for 0-24 hrs, synovial fibroblast cells were up-regulated expression of HAS2 and HAS3 gene to the highest levels at 9 hours (Figure17). HA synthesis in human synovial fibroblast cells were increased in dose and time dependent manner after cells were activated with 0-0.3  $\mu\text{g/ml}$  LPS for 0- 24 hours (Figure18).



**Figure 17.** Effects of LPS on the expression of HAS2 (A) and HAS3 (B) genes in human synovial fibroblast cells. Human synovial fibroblast cells were incubated in the absence or presence of 0.03-0.3  $\mu\text{g/ml}$  LPS for 0-24 hours. Control was cells cultured without LPS. Data shown are mean value  $\pm$  standard deviation of triplicate assay per treatment. \*, \*\* Denoted values that were significantly different from control ( $p < 0.05$  and  $p < 0.01$ , respectively).

$$\% \text{ of control} = \frac{\text{Density of sample} / \text{Density of GAPDH}}{\text{Density of untreated control} / \text{Density of GAPDH}} \times 100$$



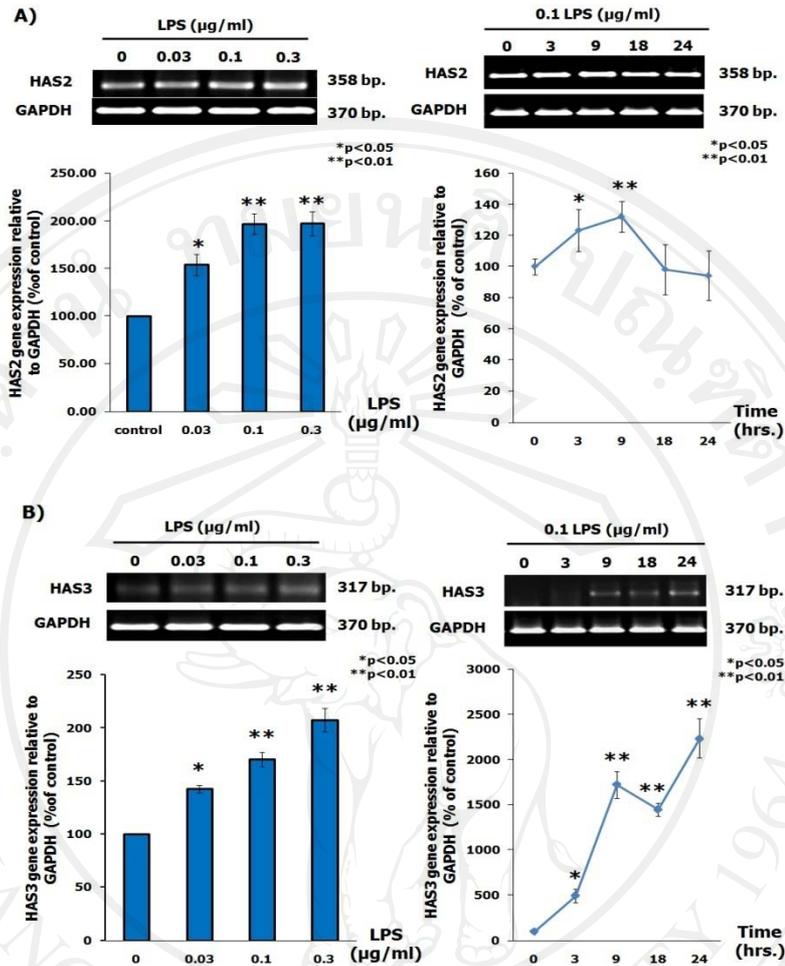
**Figure 18.** Effect of LPS on Hyaluronan synthesis in human synovial fibroblast cells. Human synovial fibroblast SW982 cell lines were incubated in the presence of 0.03-0.3 µg/ml LPS for 9 hours. Control were cells cultured without LPS. Data show are mean value  $\pm$  standard deviation of triplicate assay per treatment. \* Denoted values that were significantly different from control, ( $p < 0.05$ ).

### **3.2 Effects of Lipopolysaccharide (LPS) on human synovial fibroblast SW982 cell lines.**

Human synovial fibroblast SW982 cell lines were treated with LPS (0.03-0.3  $\mu\text{g/ml}$ ) for 0-24 hours. And then, the cell lines were harvested for investigation of HAS2 and HAS3 mRNA expression by RT-PCR. The culture medium from human synovial fibroblast SW982 cell lines treated with LPS was investigated for hyaluronan synthesis by ELISA technique.

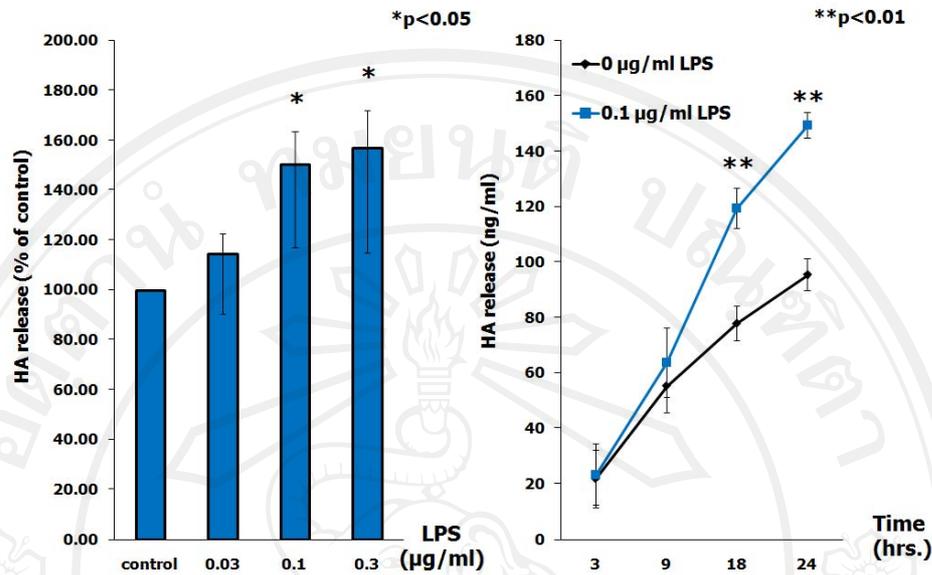
In results found that the human synovial fibroblast SW982 cell lines were induced by 0.1  $\mu\text{g/ml}$  LPS for 0-24 hours, HAS2 gene reached the highest expression at 9 hours, while HAS3 gene was found increasing through 24 hours of treatment (Figure 19). HA synthesis in human synovial fibroblast SW982 cell lines were found to be increased in dose and time dependent manner after 0-0.3  $\mu\text{g/ml}$  LPS activation for 0-24 hours (Figure 20).

In addition, the expression TLR-4 mRNA was elevated by 0-0.3  $\mu\text{g/ml}$  LPS activation (Figure 21A). Interestingly, LPS also significantly increased IL-1 $\beta$  and ICE mRNA expression in dose dependent manner for 9 hours (Figure 21B, 21C).

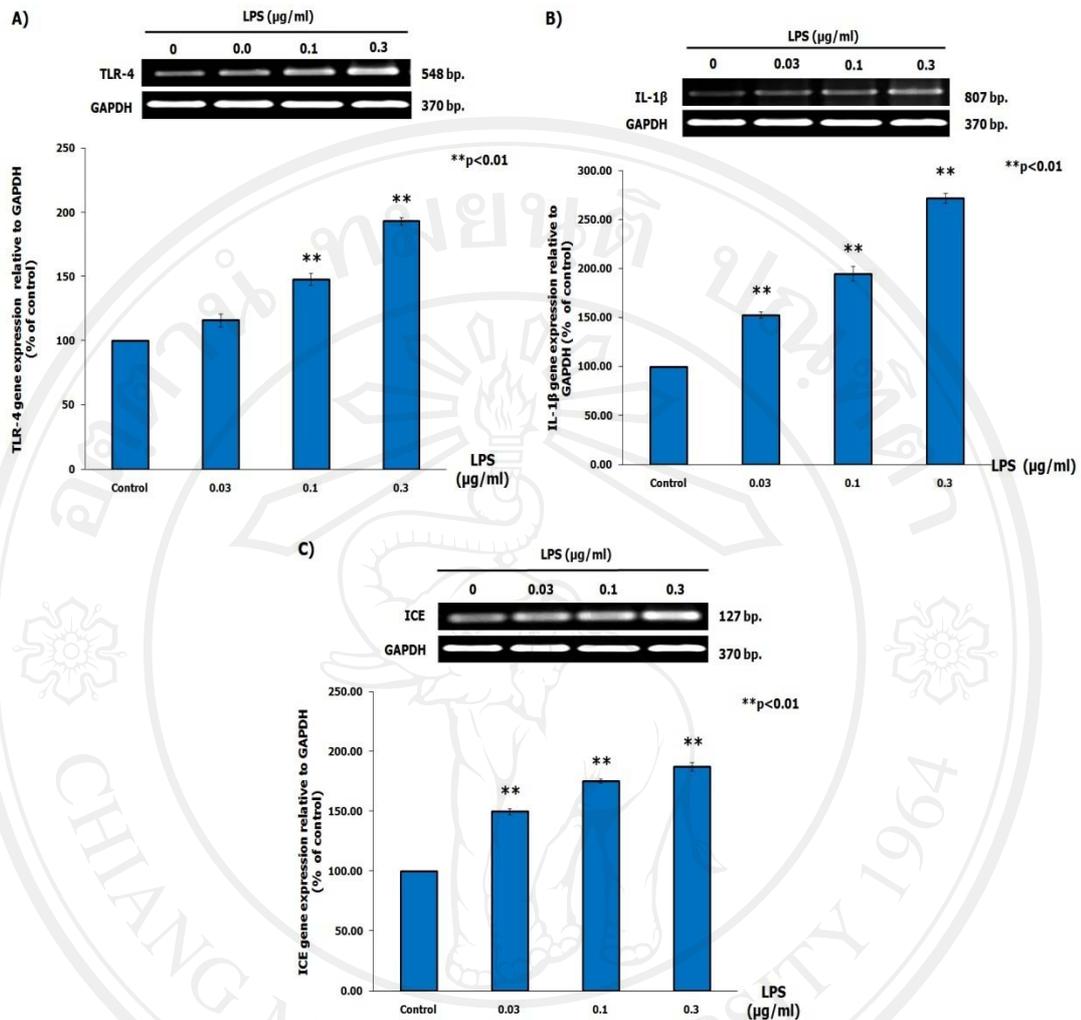


**Figure 19.** Effect of LPS on the mRNA expression of HAS2(A) and HAS3(B) in human synovial fibroblast SW982 cell lines. Human synovial fibroblast SW982 cell lines were incubated in the presence of 0.03-0.3 µg/ml LPS for 0-24 hours. Control were cells cultured without LPS. Data shown are mean value ± standard deviation of triplicate assay per treatment. \*,\*\*Denoted values that were significantly different from control (p<0.05 and p<0.01), respectively.

$$\% \text{ of control} = \frac{\text{Density of sample} / \text{Density of GAPDH}}{\text{Density of untreated control} / \text{Density of GAPDH}} \times 100$$



**Figure 20.** Effect of LPS on Hyaluronan synthesis in human synovial fibroblast SW982 cell lines. Human synovial fibroblast SW982 cell lines were incubated in the presence of 0.03-0.3 µg/ml LPS for 9 hours. Control were cells cultured without LPS. Data show are mean value  $\pm$  standard deviation of triplicate assay per treatment. \*,\*\*Denoted values that were significantly different from control, ( $p < 0.05$  and  $p < 0.01$ ) respectively.

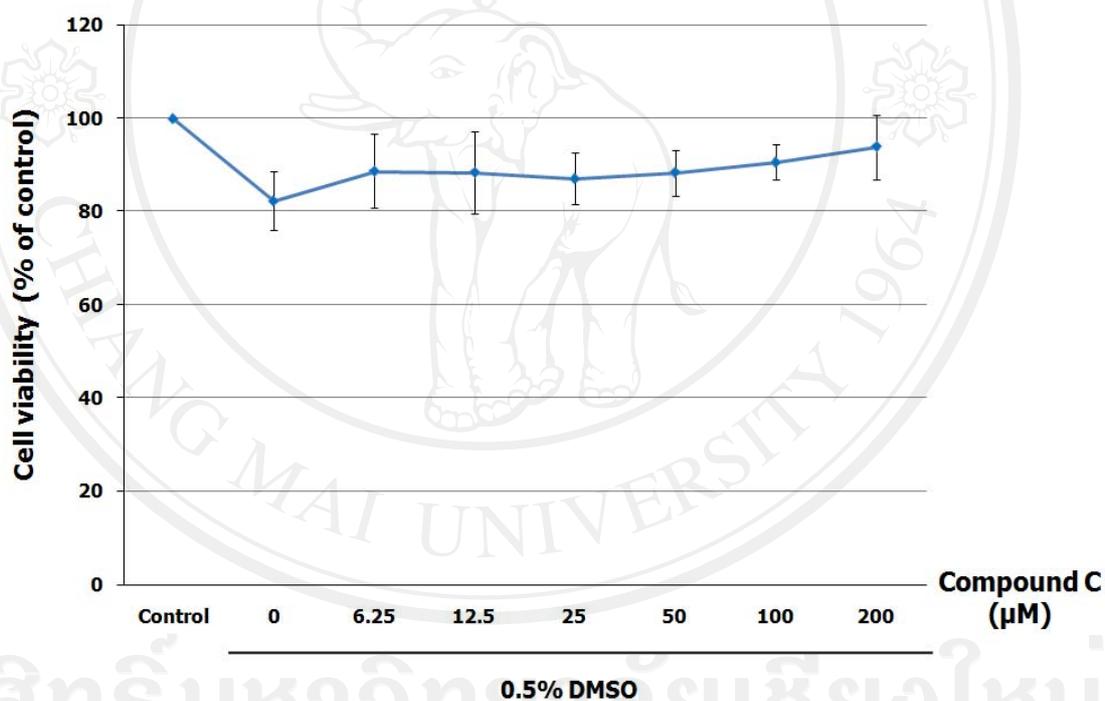


**Figure 21.** Effect of LPS on the mRNA expression of TLR-4 (A), IL-1β (B) and ICE (C) in human synovial fibroblast SW982 cell lines. Human synovial fibroblast SW982 cell lines were incubated in the presence of 0.03-0.3 μg/ml LPS for 9 hours. Control were cells cultured without LPS. Data show are mean value ± standard deviation of triplicate assay per treatment. \*\* Denoted values that were significantly different from control, (p<0.01).

$$\% \text{ of control} = \frac{\text{Density of sample} / \text{Density of GAPDH}}{\text{Density of untreated control} / \text{Density of GAPDH}} \times 100$$

### 3.3 Cytotoxic effects of compound C on human synovial fibroblast SW982 cell lines.

In order to determine cytotoxicity of compound C on SW982 cell line, cell were cultured with compound C in range of 0-200  $\mu\text{M}$ . After 24 hours of incubation, cell survivals were measured by MTT assay. Percentage of cell survival in all experiments was not different with control group (culture with media alone). This data indicated that compound C has not toxic to cell (Figure 22).



**Figure 22.** Effect of compound C on viability of human synovial fibroblast SW982 cell line. Cells were incubated with 0-200  $\mu\text{M}$  compound C (in 0.5% DMSO). Data shown are mean value  $\pm$  standard deviation of triplicate assay per treatment.

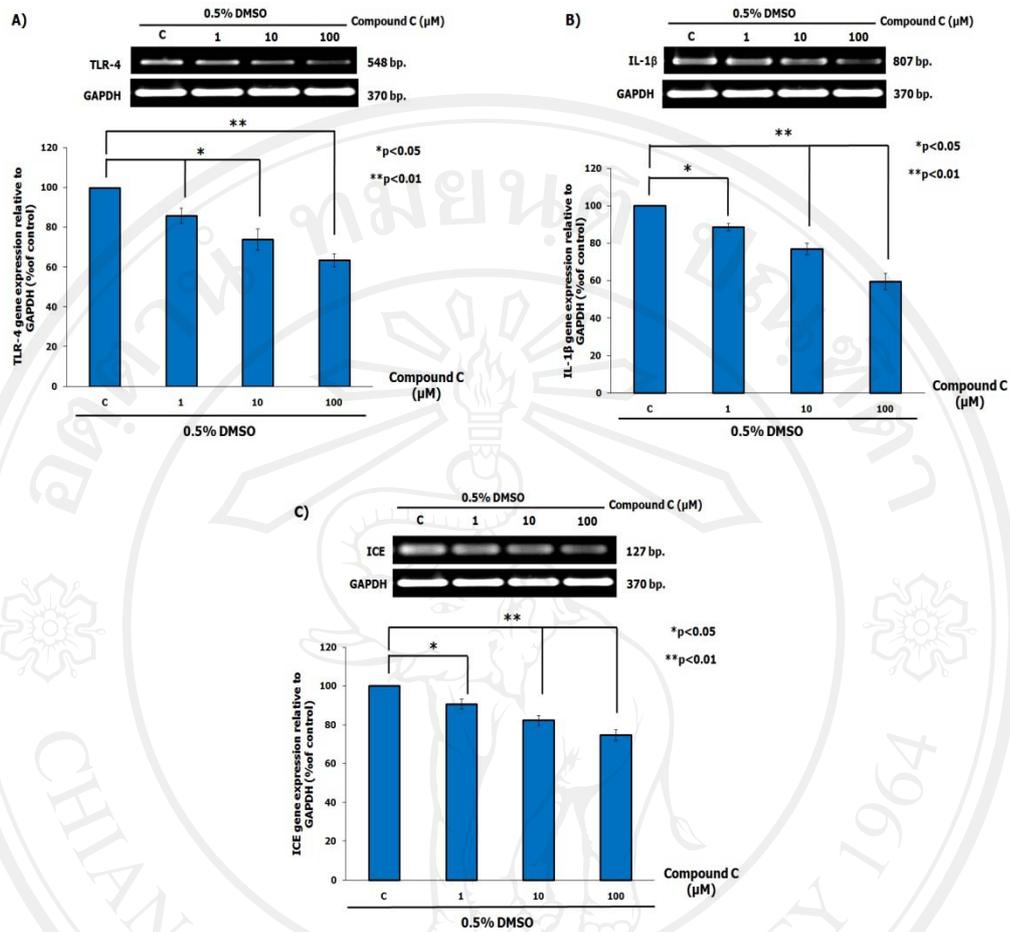
### **3.4 Effects of compound C on TLR-4, IL-1 $\beta$ , ICE, HAS2 and HAS3 gene expression in human synovial fibroblast SW982 cell line.**

Compound C, the extract from hexane fraction of Plai rhizome, inhibited the anti-inflammatory effect. In this study, the effect of compound C on HAS2, HAS3, TLR-4, IL-1 $\beta$  and ICE gene expression and HA release level were studied. Human synovial fibroblast SW982 cell line was cultured in the presence of 0-100  $\mu$ M compound C (in 0.5% DMSO) for 9 hours. After incubation, cell lines were lysed and total RNA was extracted to measure amount of TLR-4, IL-1 $\beta$ , ICE, HAS2 and HAS3 mRNA using RT-PCR. The cultured media were collected to analyze hyaluronan synthesis by ELISA technique.

Data represented that compound C in the range of 0-100  $\mu$ M reduced the expressions of TLR-4 in a dose dependent manner (Figure 23A). Concurrently, the expression of IL-1 $\beta$  and ICE genes were decreased when cells were treated with compound C (Figure 23B, 23C).

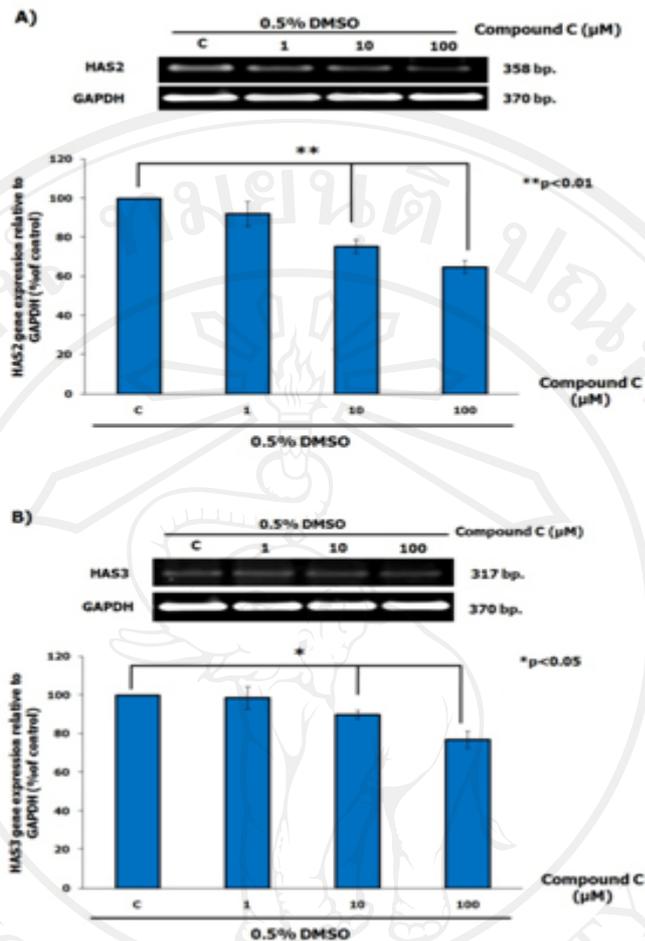
In further investigation indicated that compound C had ability to inhibit the expression of HAS2 and HAS3 mRNA from 10-100  $\mu$ M compound C (Figure 24).

Additionally, The HA levels detected by ELISA were also decreased in a dose dependent manner. It is well known that HA were synthesized by HASs activity (Figure 25).



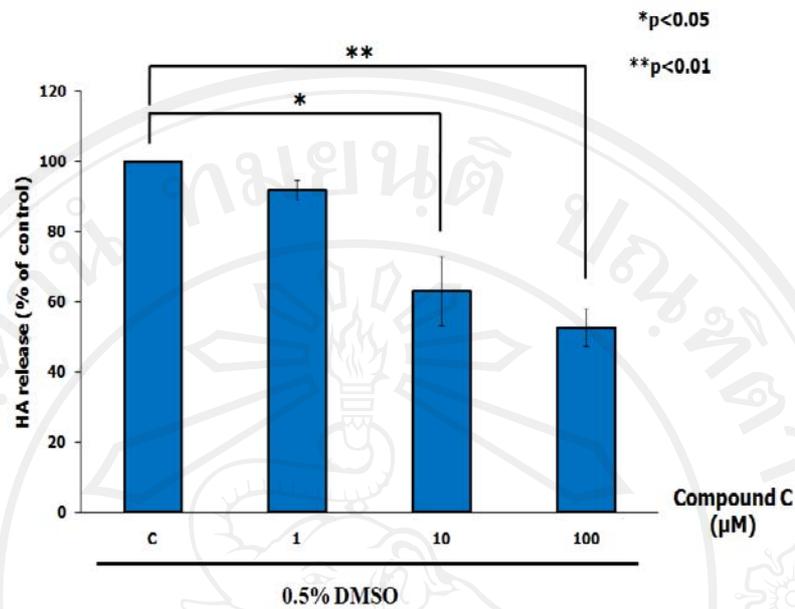
**Figure 23.** Effect of compound C on the expressions of TLR-4 (A), IL-1β (B) and ICE (C) genes in human synovial fibroblast SW982 cell lines. Human synovial fibroblast SW982 cells line were incubated in the presence of 1-100 μM compound C (in 0.5% DMSO) for 9 hours. C represent to 0.5% DMSO control were added to cells without compound C. Data shown are mean value ± standard deviation of triplicate assay per treatment. \*, \*\* Denoted values that were significantly different from 0.5% DMSO control ( $p < 0.05$  and  $p < 0.01$ ), respectively.

$$\% \text{ of control} = \frac{\text{Density of sample} / \text{Density of GAPDH}}{\text{Density of untreated control} / \text{Density of GAPDH}} \times 100$$



**Figure 24.** Effect of compound C on the expressions of HAS2 (A) and HAS3 (B) genes in human synovial fibroblast SW982 cell lines. Human synovial fibroblast SW982 cells line were incubated in the presence of 1-100  $\mu\text{M}$  compound C (in 0.5% DMSO) for 9 hours. C represent to 0.5% DMSO control were added to cells without compound C. Data shown are mean value  $\pm$  standard deviation of triplicate assay per treatment. \*, \*\* Denoted values that were significantly different from 0.5% DMSO control ( $p < 0.05$  and  $p < 0.01$ ), respectively.

$$\% \text{ of control} = \frac{\text{Density of sample} / \text{Density of GAPDH}}{\text{Density of untreated control} / \text{Density of GAPDH}} \times 100$$



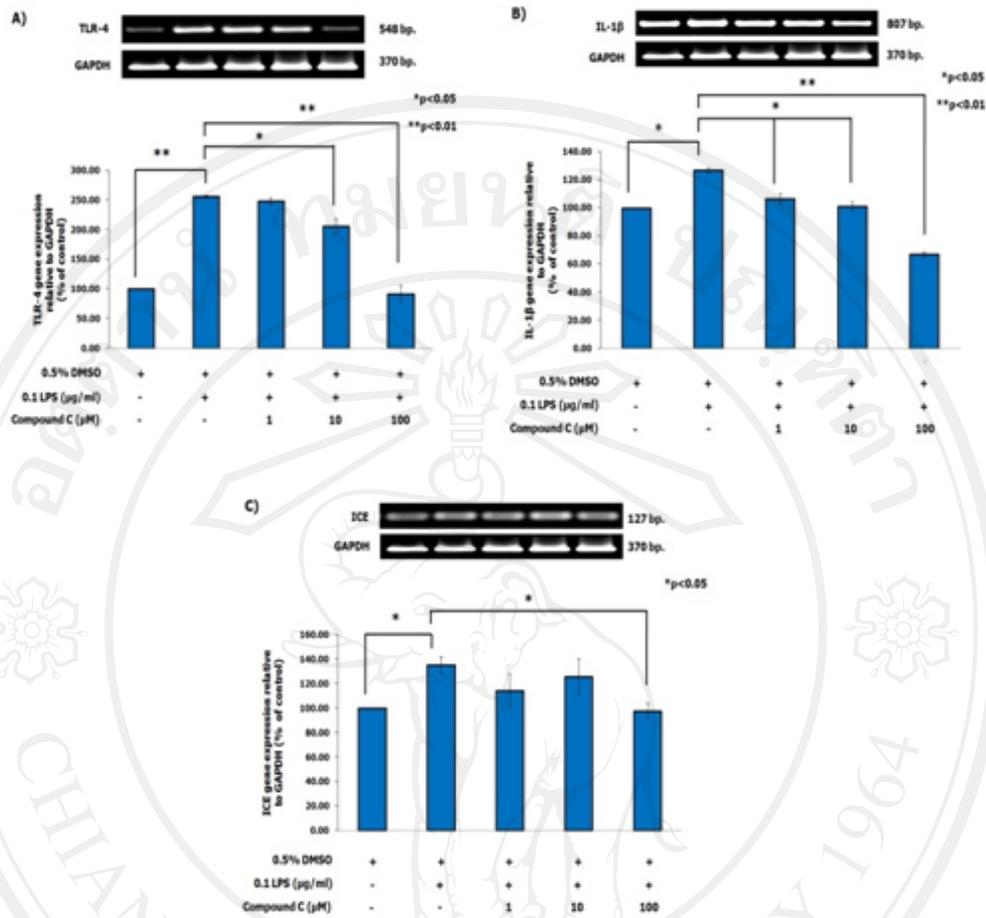
**Figure 25.** Effect of compound C on Hyaluronan synthesis in human synovial fibroblast SW982 cell lines. Human synovial fibroblast Human synovial fibroblast SW982 cell lines were incubated in the presence of 1-100  $\mu\text{M}$  compound C (in 0.5% DMSO) for 9 hours. C represent to 0.5% DMSO control were added to cells without compound C. Control were cells cultured without LPS. Data shown are mean value  $\pm$  standard deviation of triplicate assay per treatment. \*,\*\* Denoted values that were significantly different from 0.5% DMSO control ( $p<0.05$  and  $p<0.01$ ), respectively.

### **3.5 Inhibitory effects of compound C on TLR-4, IL-1 $\beta$ , ICE, HAS2 and HAS3 gene expression in human synovial fibroblast SW982 cell line treated with lipopolysaccharide (LPS).**

In order to investigate whether LPS-induced TLR-4, IL-1 $\beta$ , ICE, HAS2 and HAS3 mRNA expression and HA synthesis in human synovial fibroblast SW982 cell lines were inhibited by compound C. Cells were co-treated with 0-100  $\mu$ M compound C (in 0.5% DMSO) and 0.1  $\mu$ g/ml LPS for 9 hours. Then, the cultured medium were collected to analyze hyaluronan synthesis using ELISA technique, and cells were harvested for examinations of HAS2, HAS3 and TLR-4 mRNA expressions by RT-PCR.

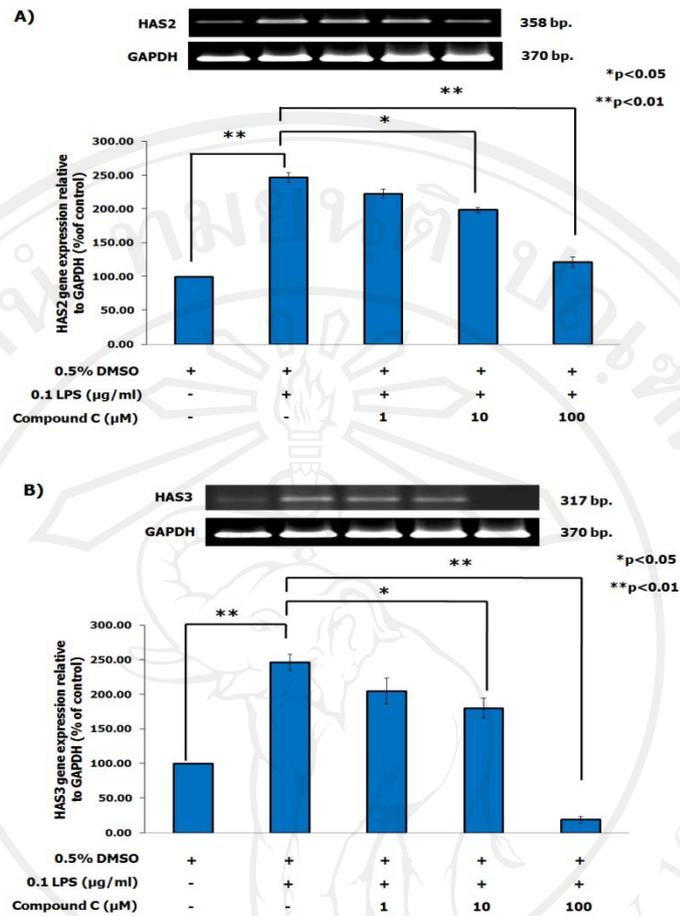
These results demonstrated that compound C inhibited LPS-induced gene expression of TLR-4 (Figure 26A). Furthermore, the co-stimulation between compound C with LPS suppressed IL-1 $\beta$  and ICE gene expression (Figure 26B, 26C).

Inhibitory effect of compound C on HAS2 and HAS3 gene expression treated with LPS in SW982 cell line was further studied. Expression of HAS2 and HAS3 gene were reduced by compound C in presence of LPS (Figure 27). Consistently, HA release represented HASs activity in culture medium was found to decrease when cells were stimulated by LPS (Figure 28).



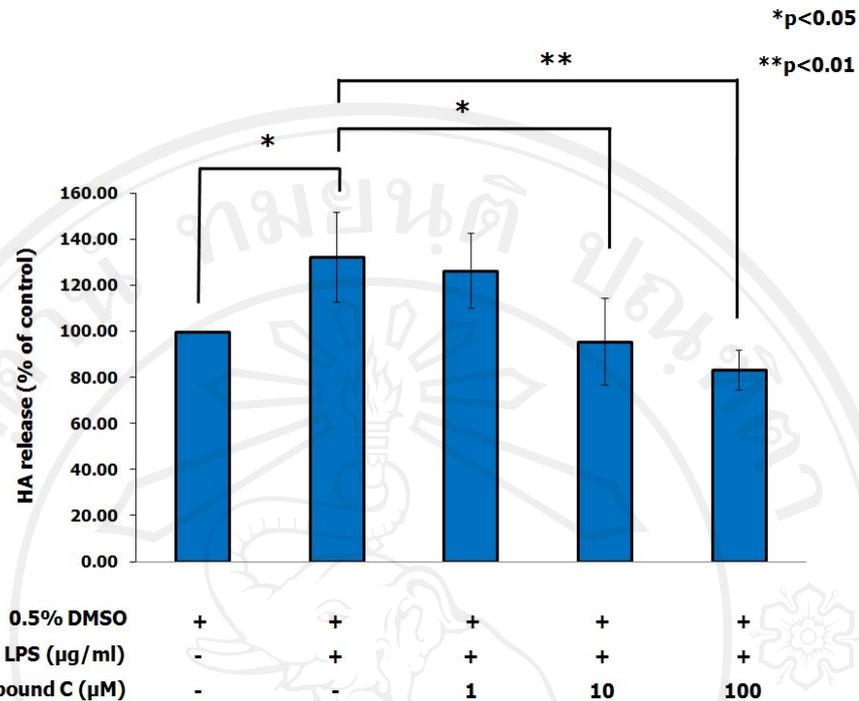
**Figure 26.** Effect of compound C on the mRNA expression of TLR-4 (A), IL-1 $\beta$  (B) and ICE (C) in human synovial fibroblast SW982 cells line treated with 0.1  $\mu$ g/ml LPS. Human synovial fibroblast SW982 cells line were co-treated with 1-100  $\mu$ M compound C (in 0.5% DMSO) with 0.1  $\mu$ g/ml LPS for 9 hours. 0.5% DMSO control and 0.1  $\mu$ g/ml LPS stimulation were added to cells without compound C. Data shown are mean value  $\pm$  standard deviation of triplicate assay per treatment. \*, \*\* Denoted values that were significantly different ( $p < 0.05$  and  $p < 0.01$ ), respectively.

$$\% \text{ of control} = \frac{\text{Density of sample/Density of GAPDH}}{\text{Density of 0.5\% DMSO control /Density of GAPDH}} \times 100$$



**Figure 27.** Effect of compound C on the mRNA expression of HAS2 (A) and HAS3 (B) in human synovial fibroblast SW982 cells line treated with 0.1 µg/ml LPS. Human synovial fibroblast SW982 cells line were co-treated with 1-100 µM compound C (in 0.5% DMSO) with 0.1 µg/ml LPS for 9 hours. 0.5% DMSO control and 0.1 µg/ml LPS stimulation were added to cells without compound C. Data shown are mean value ± standard deviation of triplicate assay per treatment. \*, \*\* Denoted values that were significantly different ( $p < 0.05$  and  $p < 0.01$ ), respectively.

$$\% \text{ of control} = \frac{\text{Density of sample} / \text{Density of GAPDH}}{\text{Density of 0.5\% DMSO control} / \text{Density of GAPDH}} \times 100$$



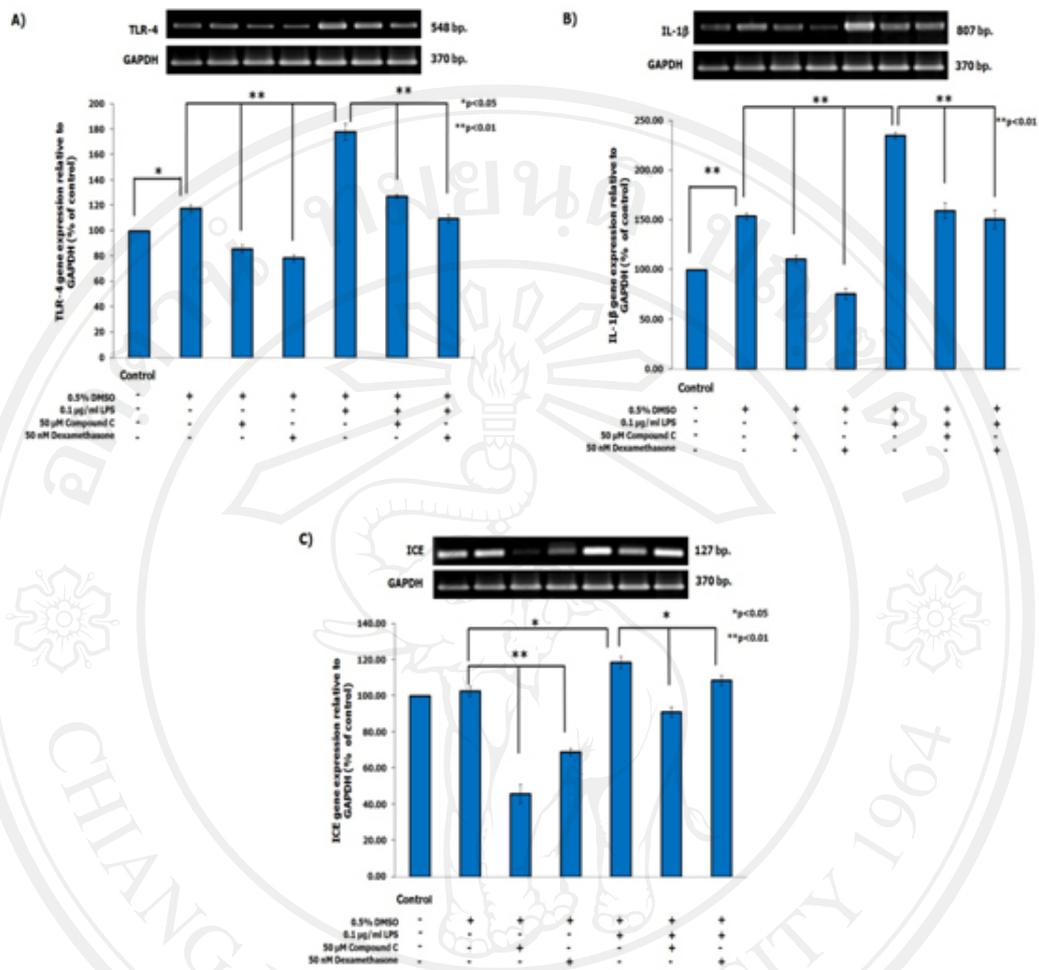
**Figure 28.** Effect of compound C on hyaluronan synthesis in human synovial fibroblast SW982 cell lines after 0.1  $\mu\text{g/ml}$  LPS stimulation. Human synovial fibroblast SW982 cell lines were co-treated with 1-100  $\mu\text{M}$  compound C (in 0.5% DMSO) with 0.1  $\mu\text{g/ml}$  LPS for 9 hours. 0.5% DMSO control and 0.1  $\mu\text{g/ml}$  LPS stimulation were added to cells without compound C. Data shown are mean value  $\pm$  standard deviation of triplicate assay per treatment. \*, \*\* Denoted values that were significantly different ( $p<0.05$  and  $p<0.01$ ), respectively.

**3.6 Inhibitory effect of compound C and dexamethasone on TLR-4, IL-1 $\beta$ , ICE, HAS2 and HAS3 human synovial fibroblast SW982 cell lines treated with lipopolysaccharide (LPS).**

In order to find out the effect of 50  $\mu$ M compound C (in 0.5% DMSO) and 50 nM dexamethasone (in 0.5% DMSO), cells were stimulated with or without 0.1  $\mu$ g/ml LPS in the presence or absence of compound C and dexamethasone for 9 hours. Dexamethasone was considered as drug for RA. Thus, we examined the ability of compound C compared with dexamethasone. Then, the releases of HA were investigated in the cultured media by HA ELISA. The expressions of TLR-4, IL-1 $\beta$ , ICE, HAS2 and HAS3 were measured from harvested cells using RT-PCR.

The results show that both 50  $\mu$ M compound C and 50 nM dexamethasone reduced the mRNA expression of TLR-4, IL-1 $\beta$  and ICE after stimulation with or without 0.1  $\mu$ g/ml LPS (Figure 29).

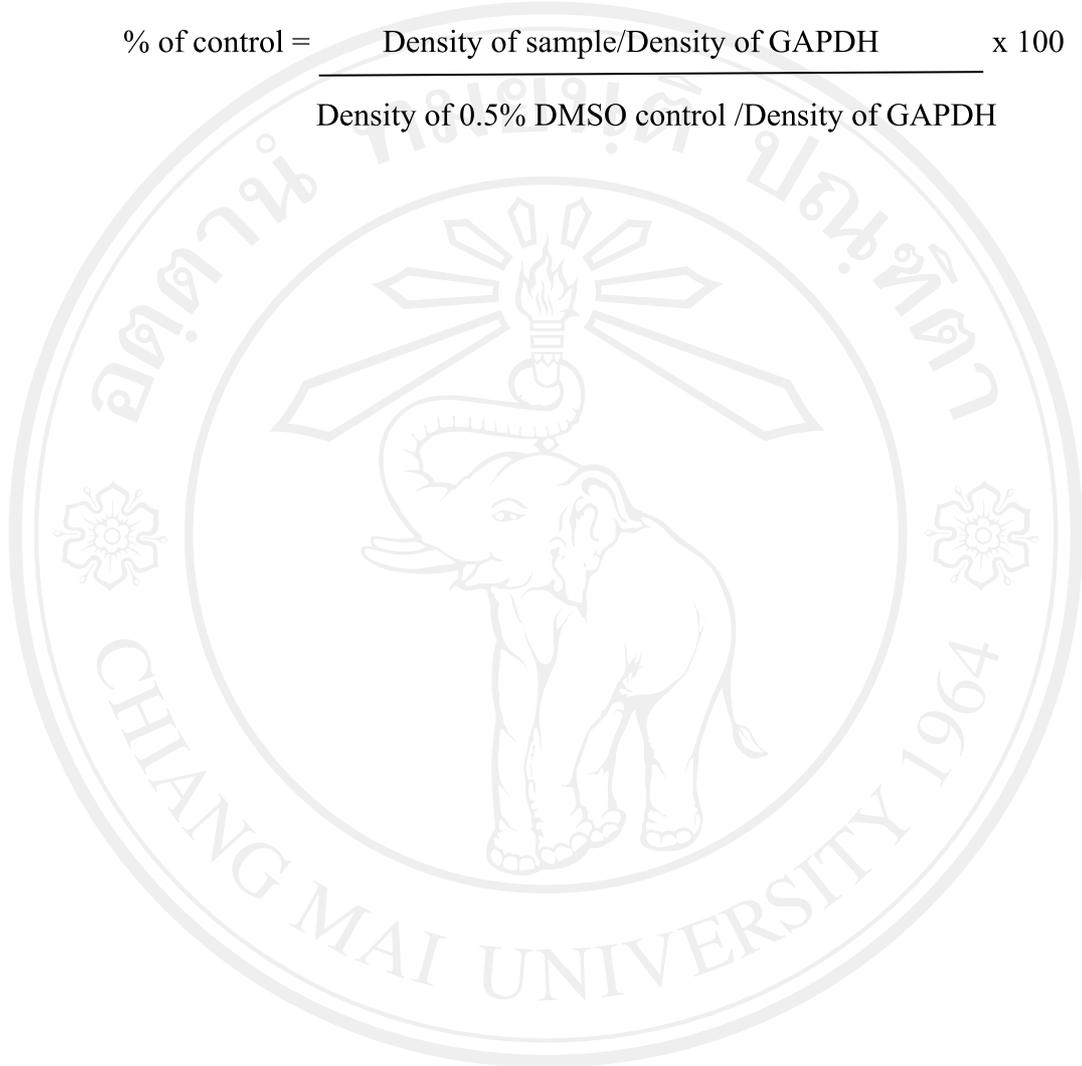
Interestingly, compound C incubated SW982 cell lines in the absence and presence of LPS were found the reduction of HAS2 and HAS3 mRNA levels when compared with Dexamethasone. (Figure 30) These results agreed with the reduction of HA release which represented HASs activity (Figure 31).



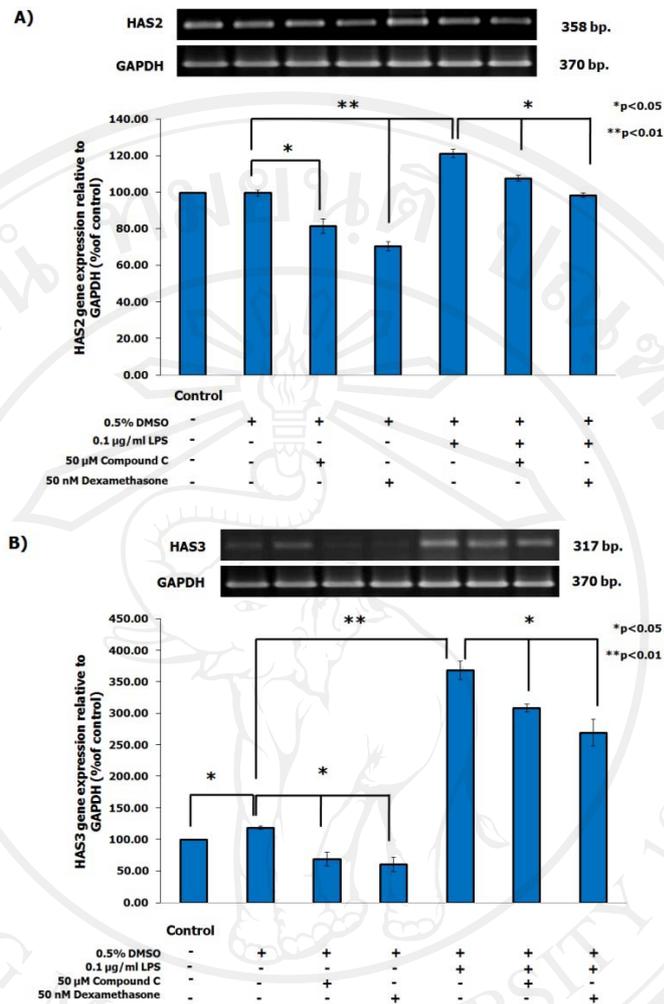
**Figure 29.** Effects of compound C and dexamethasone on the mRNA expressions of TLR-4 (A), IL-1 $\beta$  (B) and ICE (C) in human synovial fibroblast SW982 cell line in the presence and absence 0.1  $\mu$ g/ml LPS. Human synovial fibroblast SW982 cells line were cultured with 50  $\mu$ M compound C (in 0.5% DMSO) or 50 nM dexamethasone (in 0.5% DMSO) after they were stimulated with or without 0.1  $\mu$ g/ml LPS for 9 hours. Control, 0.5% DMSO control and 0.1  $\mu$ g/ml LPS stimulation were cells cultured without compound C. Data shown are mean value  $\pm$  standard deviation of

triplicate assay per treatment. \*, \*\* Denoted values that were significantly different (p<0.05 and p<0.01), respectively.

$$\% \text{ of control} = \frac{\text{Density of sample/Density of GAPDH}}{\text{Density of 0.5\% DMSO control /Density of GAPDH}} \times 100$$



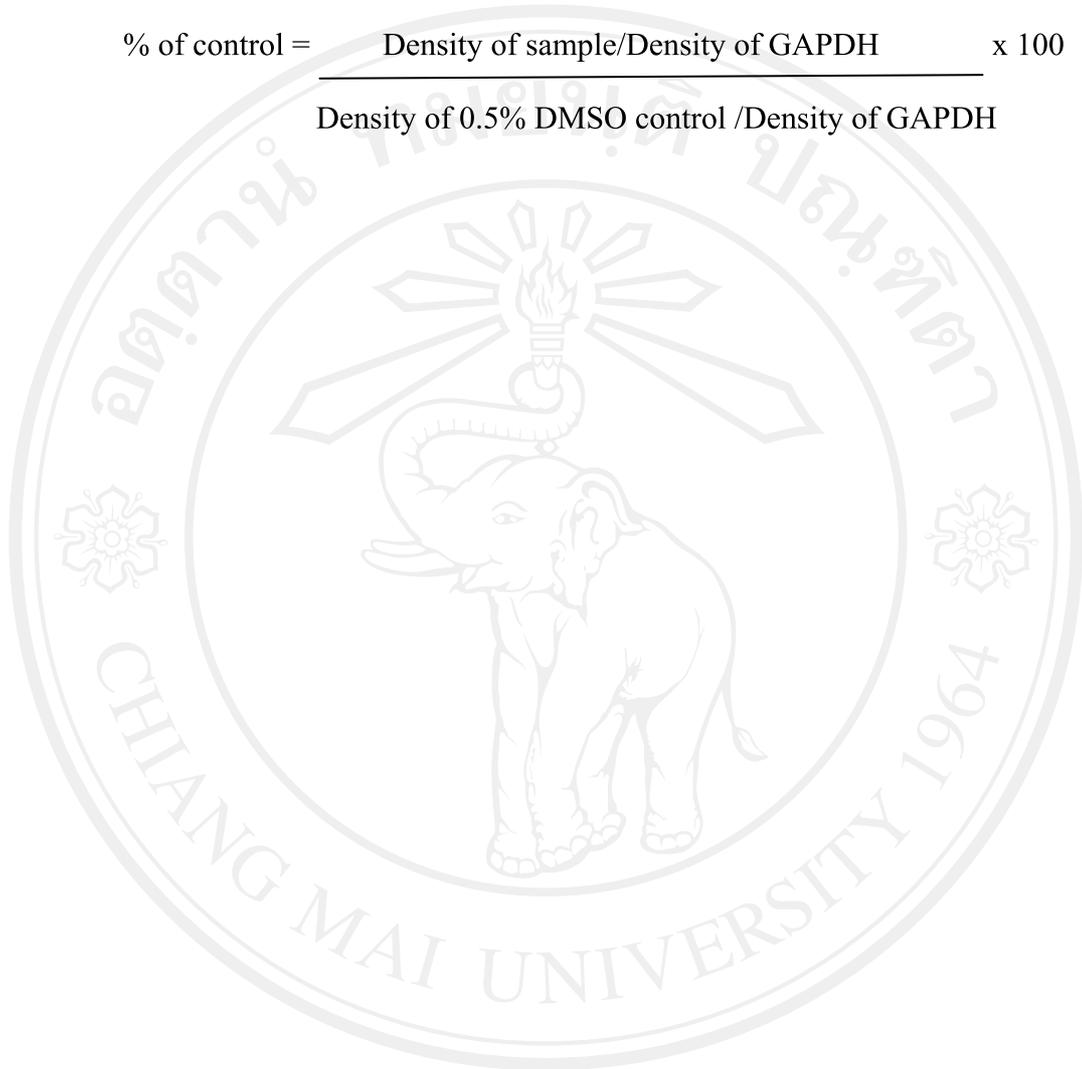
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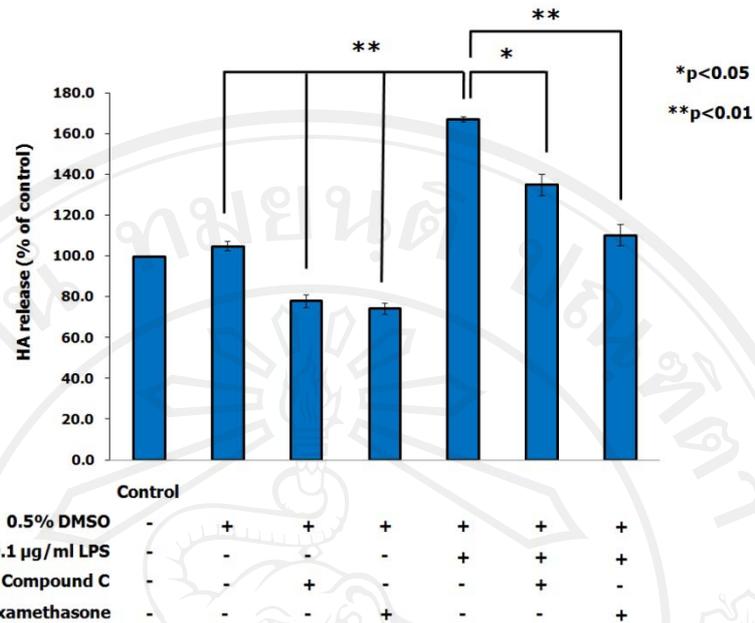
**Figure 30.** Effects of compound C and dexamethasone on the mRNA expressions of HAS2 (A) and HAS3 (B) in human synovial fibroblast SW982 cell line in the presence and absence 0.1 µg/ml LPS. Human synovial fibroblast SW982 cells line were cultured with 50 µM compound C (in 0.5% DMSO) or 50 nM dexamethasone (in 0.5% DMSO) after they were stimulated with or without 0.1 µg/ml LPS for 9 hours. Control, 0.5% DMSO control and 0.1 µg/ml LPS stimulation were cells cultured without compound C. Data shown are mean value ± standard deviation of

triplicate assay per treatment. \*, \*\* Denoted values that were significantly different (p<0.05 and p<0.01), respectively.

$$\% \text{ of control} = \frac{\text{Density of sample/Density of GAPDH}}{\text{Density of 0.5\% DMSO control /Density of GAPDH}} \times 100$$



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**Figure 31.** Effect of compound C and dexamethasone on Hyaluronan synthesis in human synovial fibroblast SW982 cell line in presence and absence of 0.1 µg/ml LPS. Human synovial fibroblast SW982 cell lines were cultured in 50 µM compound C (in 0.5% DMSO) or 50 nM dexamethasone (in 0.5% DMSO) after they were stimulated with or without 0.1 µg/ml LPS for 9 hours. Control, 0.5% DMSO control and 0.1 µg/ml LPS stimulation were cells cultured without compound C. Data shown are mean value ± standard deviation of triplicate assay per treatment. \*, \*\* Denoted values that were significantly different, (p<0.05 and p<0.01, respectively)