## CHAPTER III

## RESULTS

### 3.1 Effects of *B. pandurata* extracts, and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of hyaluronan (HA) from oral fibroblast medium.

The oral fibroblasts were cultured in serum-free DMEM. Then cultured mediums were treated with *B. pandurata* extracts (methanol, acetone, ethyl acetate and hexane) at dose 2, 10 and 50μg/ml for 24 hours. Hyaluronan in the cultured medium was analyzed by ELISA assay. The results showed that the methanol and acetone extracts were able to inhibit the release of HA into the culture media in dose dependent manner at dose 10-50μg/ml (about 34.4-37.2 % and 5.7-78.2% respectively). Hexane significantly reduced HA level at dose 10μg/ml (about 28.3 %), the result shown in Figure 10. The morphology of cells was investigated by microscopy .The result showed that 50 μg/ml of hexane and ethyl acetate extracts changed phenotype of the cells as compared to the untreated primary cells (Figure 11) .

*B. pandurata* extracts were added to the medium of oral fibroblasts at dose 10, 50μg/ml in with or without of 1-2O-tetradecanoyl-phorbol-13-acetate (TPA) at dose 100ng/ml. After 24hours of treatment, the culture media were collected for analysis. TPA was found to dramatically increase HA level when compared with the control. *B. pandurata* extracts namely methanol and acetone significantly reduced HA level induced by TPA in dose dependent manner (10, 50μg/ml). Hexane and ethyl acetate significantly reduced HA level induced by TPA at 10μg/ml, the result shown in Figure 12. But 50 μg/ml of hexane and ethyl acetate extracts toxic to cell, the result shown in Figure 11. Therefore in the next experiments, this study did not use these concentrations.

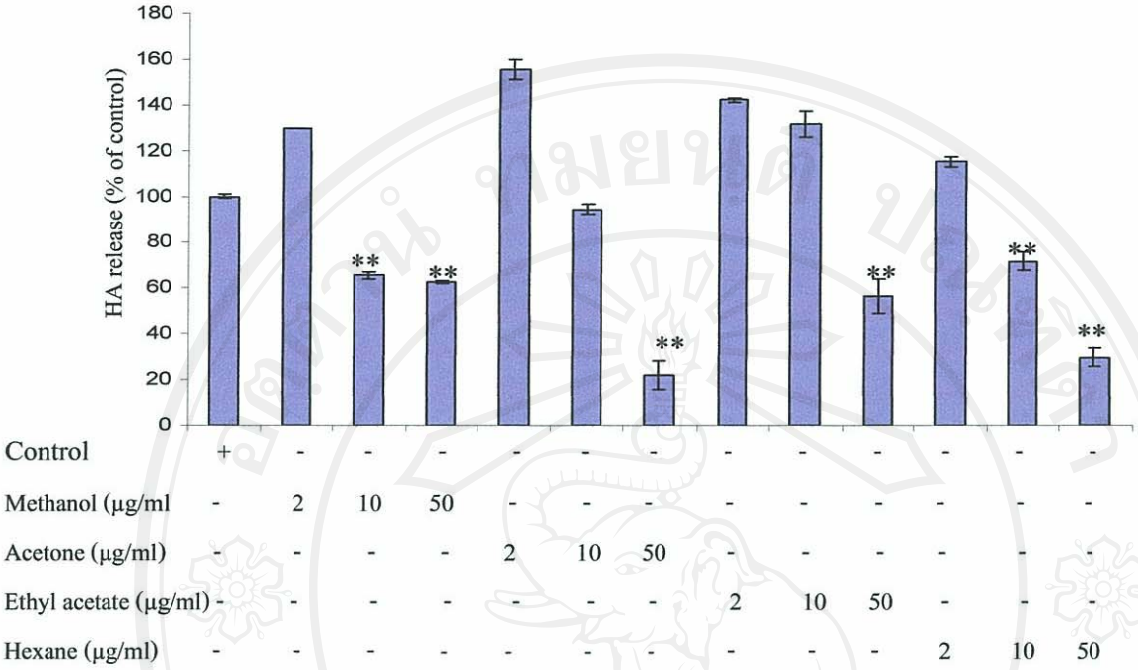
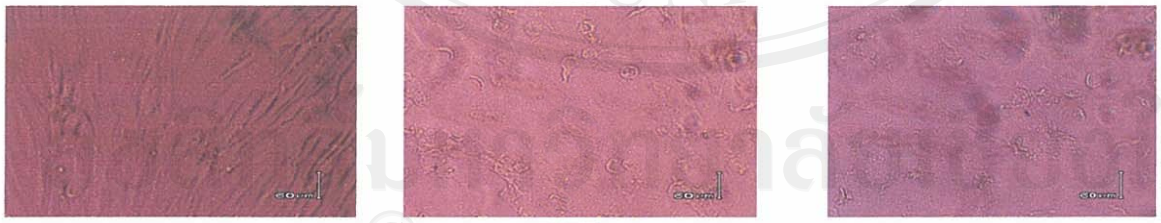



**Figure 10.** Inhibitory effect of various concentration of *B. pandurata* extracts on the release of HA from oral fibroblast medium. Cells were treated with methanol, hexane, acetone and ethyl acetate extracts added at doses 2, 10, 50 µg/ml and solvent control (DMSO). Data are the mean values ± standard deviation of triplicate per treatment. ** Denoted values that was significantly different from control ($p < 0.01$).



A. Normal control B. Ethyl acetate extract 50 µg/ml C. Hexane extract 50 µg/ml

**Figure 11.** The effect of 50 µg/ml hexane and ethyl acetate extracts of *B. pandurata* on the phenotype of oral fibroblasts.

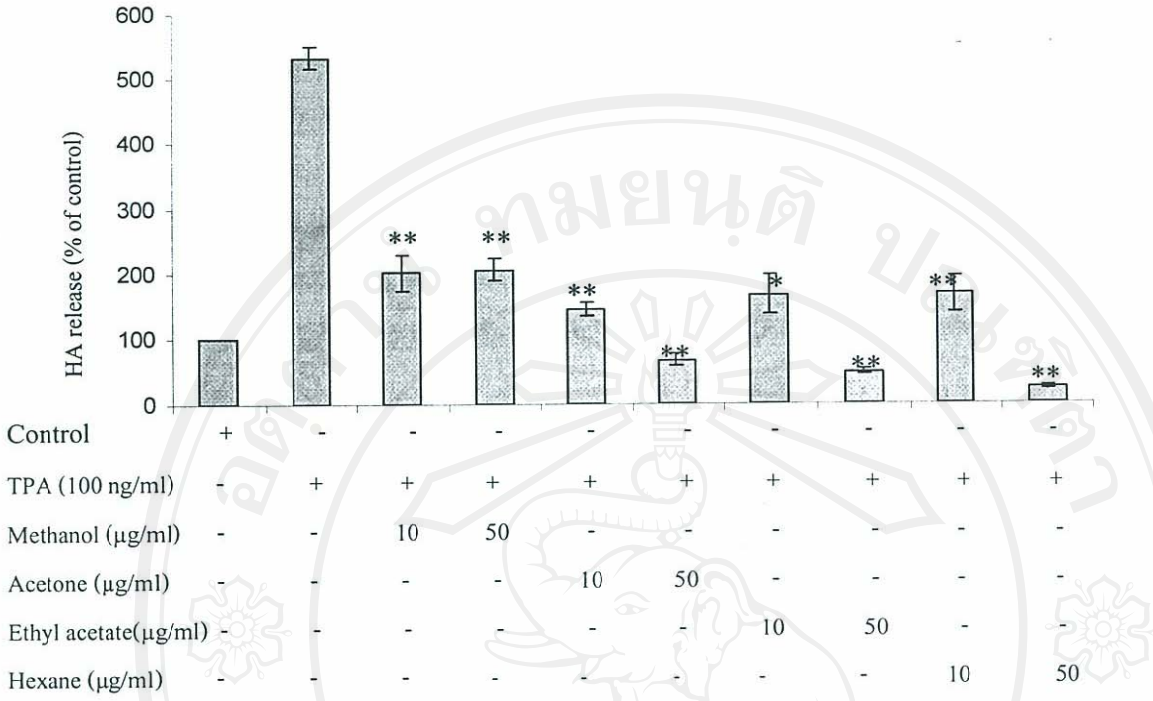



**Figure 12.** Inhibitory effect of various concentration of *B. pandurata* extracts on the release of HA induced by TPA from oral fibroblast medium. Cells were treated with 100 ng/ml TPA and methanol, hexane, acetone and ethyl acetate extract added at doses 10, 50 µg/ml, and solvent control (DMSO) and 100 ng/ml TPA-treated control. Data shown are mean value ±standard deviation of triplicate assay per treatment. *, ** Denoted values that were significantly different from TPA-treated control, ($p<0.05$) and ($p<0.01$) respectively.

**3.2 Effects of pinocembrin, pinostrobin, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and interleukin-1β (IL-1β) on the level of hyaluronan (HA) in oral fibroblast medium .**

To study the effect of flavonoids isolated from *B. pandurata* (pinostrobin and pinocembrin) on the level of HA, oral fibroblast were treated with various concentrations of pinocembrin and pinostrobin at dose 3 ,10, 30 µg/ml overnight. The release of HA was investigated in the culture medium by ELISA assay. The results showed that the releases of HA was inhibited by pinocembrin and pinostrobin from doses 10 µg/ml in dose dependent manner (Figure 13).

In the present of stimulators, TPA or IL-1ß, HA levels in the culture medium were found to dramatically increase (Figure 14 and 15), however, these effects were inhibited when co-treated with pinocembrin or pinostrobin from 3 µg/ml in dose dependent manner. At the same concentration, pinocembrin showed stronger inhibitory effect than pinostrobin.

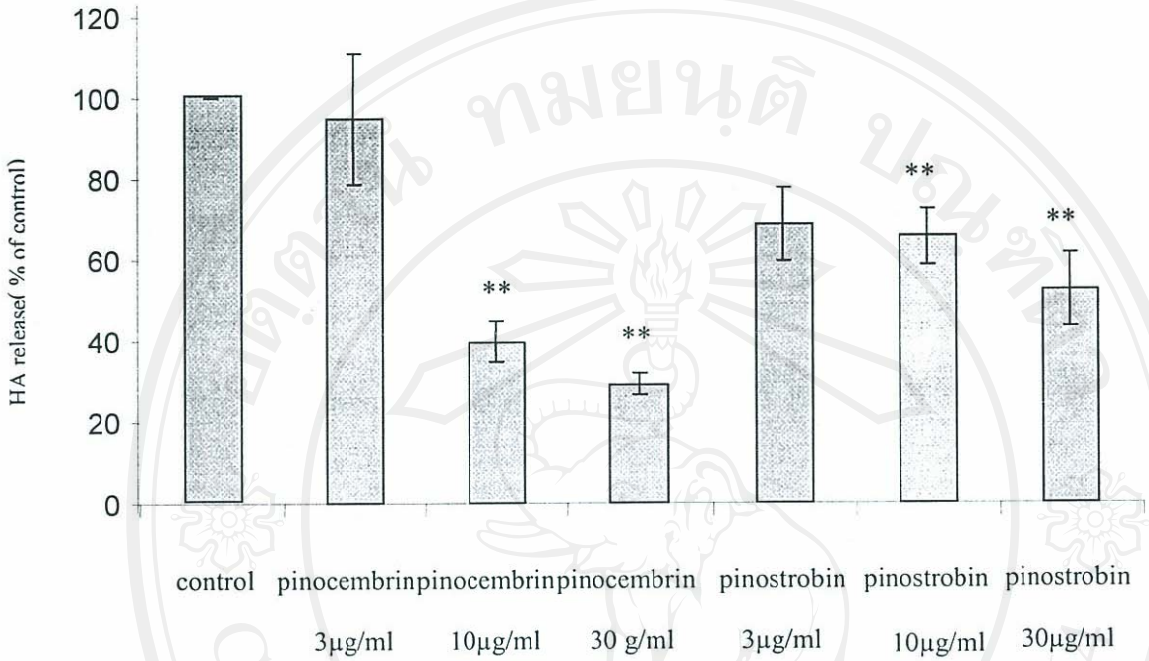



**Figure13.** Inhibitory effect of various concentration of pinocembrin and pinostrobin on the release of HA from oral fibroblast medium. Cells were treated with pinocembrin and pinostrobin added at doses 3, 10, 30 µg/ml and solvent control (DMSO). Data are the mean values ± standard deviation of triplicate per treatment. ** Denoted values that was significantly different from control ($p < 0.01$).

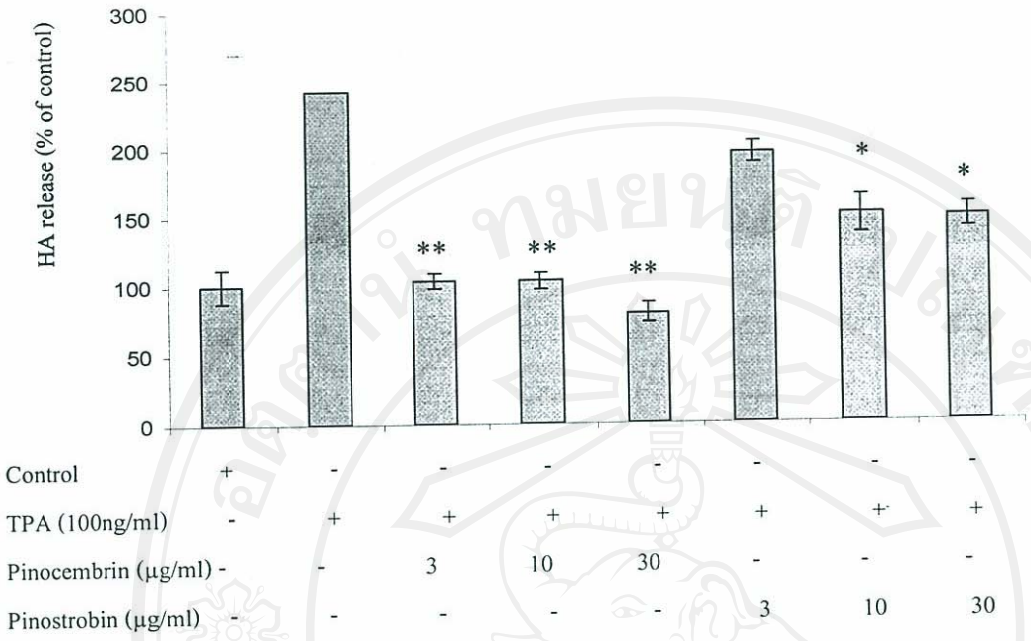



**Figure 14.** Inhibitory effect of various concentration of pinocembrin and pinostrobin on the release of HA induced by TPA from oral fibroblast medium. Cells were treated with 100 ng/ml TPA and pinocembrin and pinostrobin added at doses 3, 10, 30µg/ml, solvent control (DMSO) and 100 ng/ml TPA-treated control. Data shown are mean value ±standard deviation of triplicate assay per treatment. *,** Denoted values that were significantly different from TPA-treated control, ($p<0.05$) and ($p<0.01$) respectively.

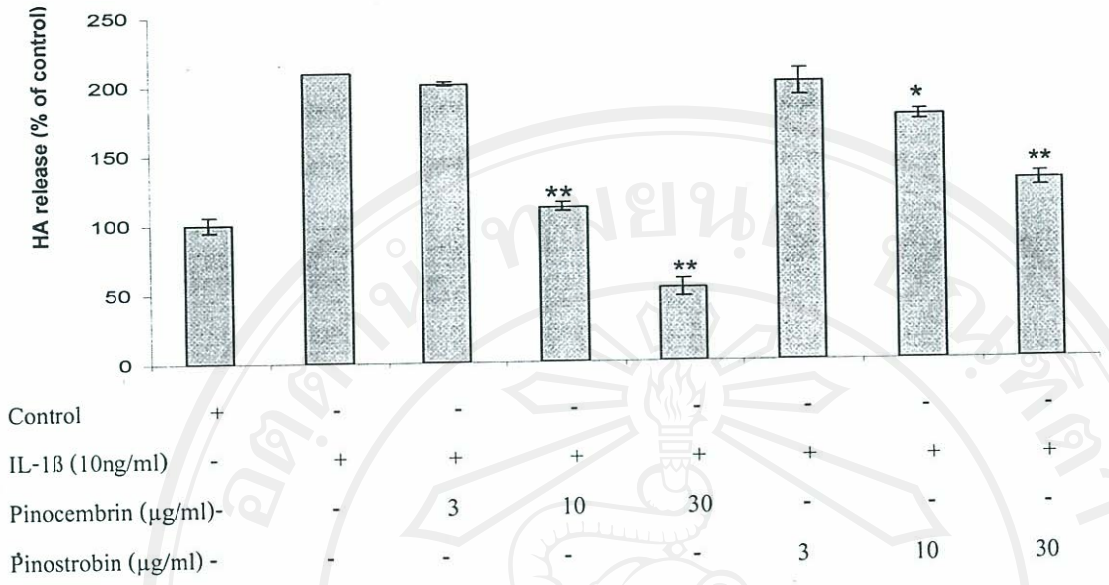



**Figure 15**. Inhibitory effect of various concentration of pinocembrin and pinostrobin on the release of HA induced by IL-1β from oral fibroblast medium. Cells were treated with 10ng/ml IL-1β and pinocembrin and pinostrobin added at doses 3, 10, 30µg/ml, solvent control (DMSO) and 10 ng/ml IL-1β-treated control. Data shown are mean value ±standard deviation of triplicate assay per treatment. *, ** Denoted values that were significantly different from IL-1β-treated control, ($p<0.05$) and ($p<0.01$) respectively.

**3.3 Effects of flavonoid derivatives on the level of hyaluronan in oral fibroblast medium .**

Flavonoids are a group of natural product with promising biological activities, found ubiquitously implants. The flavonoids have a remarkable reputation to possess anti-allergic, anti hemorrhagic, antiviral activities and anti-inflammation. In this study, eight flavonoid derivatives such as β-napthoxyflavone, 3-hydroxy-6-methoxyflavone, 2'-methoxyflavone, flavone, 5-methoxyflavone, 7-hydroxyflavone, 5-hydroxyflavone and 3-hydroxy-7-methoxyflavone) were used to compare the effect on the level of hyaluronan.

Oral fibroblasts were cultured in present of 10 g/ml flavonoid derivatives and isolated flavonoids from *B. pandurata* (pinostrobin and pinocembrin) to examine the effect on HA level. Then, the release of HA was investigated in the culture media. As shown in Figure 16, 5-methoxyflavone, flavone, pinocembrin and pinostrobin were able to inhibit the release of HA into the culture media, while 2'-methoxyflavone, 7-hydroxyflavone, 5-hydroxyflavone and β-napthoxyflavone had no significant changes in HA level. In contrast, 3-hydroxy-6-methoxyflavone and 3-hydroxy-7-methoxyflavone show activity to induce the release of HA into the culture media compare to the untreated control.

Oral fibroblasts were cultured with 100μg/ml TPA or 10ng/ml IL-1ß in absence or presence of 10μg/ml flavonoid derivatives and isolated flavonoids from *B. pandurata* to examine the effect on the level of hyaluronan. As shown in Figure 17 and 18, 5-methoxyflavone, 2'-methoxyflavone, 5-hydroxyflavone flavone, ß-napthoxyflavone, pinocembrin and pinostrobin were able to reduce HA stimulated by TPA and IL-1ß. On the other hand, 3-hydroxy-6-methoxyflavone, 3-hydroxy-7-methoxyflavone and 7-hydroxyflavone were able to induce the release of HA into the culture media. Pinocembrin showed the highest inhibitory effect on the release of HA compared to the other flavonoid derivatives.

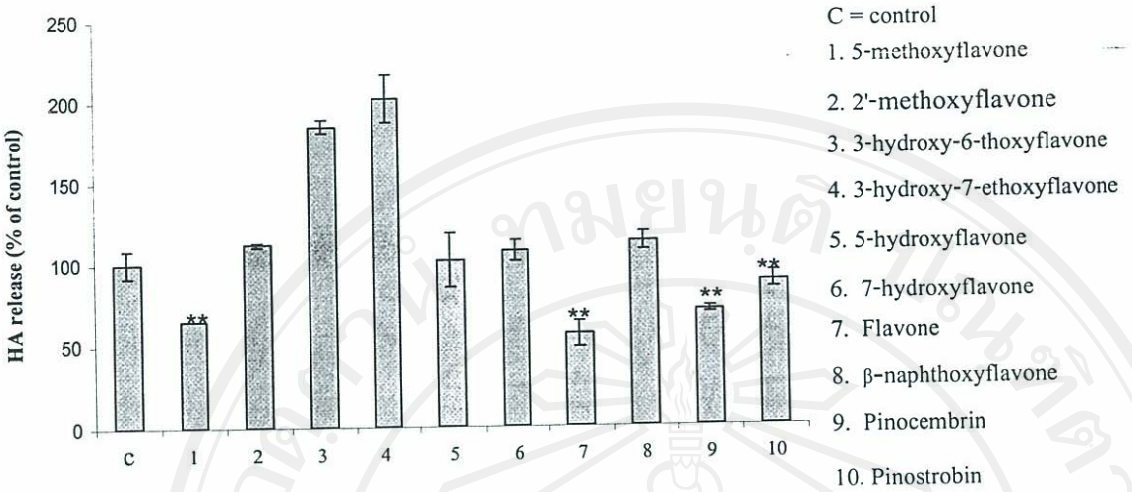



**Figure16.** Inhibitory effect of pinocembrin, pinostrobin and flavonoid derivatives on the release of HA from oral fibroblast medium. Cells were treated with 10μg/ml pinocembrin, pinostrobin, flavonoid derivatives and solvent control (DMSO). Data are the mean values ± standard deviation of triplicate per treatment. ** Denoted values that was significantly different from control ($p < 0.01$).

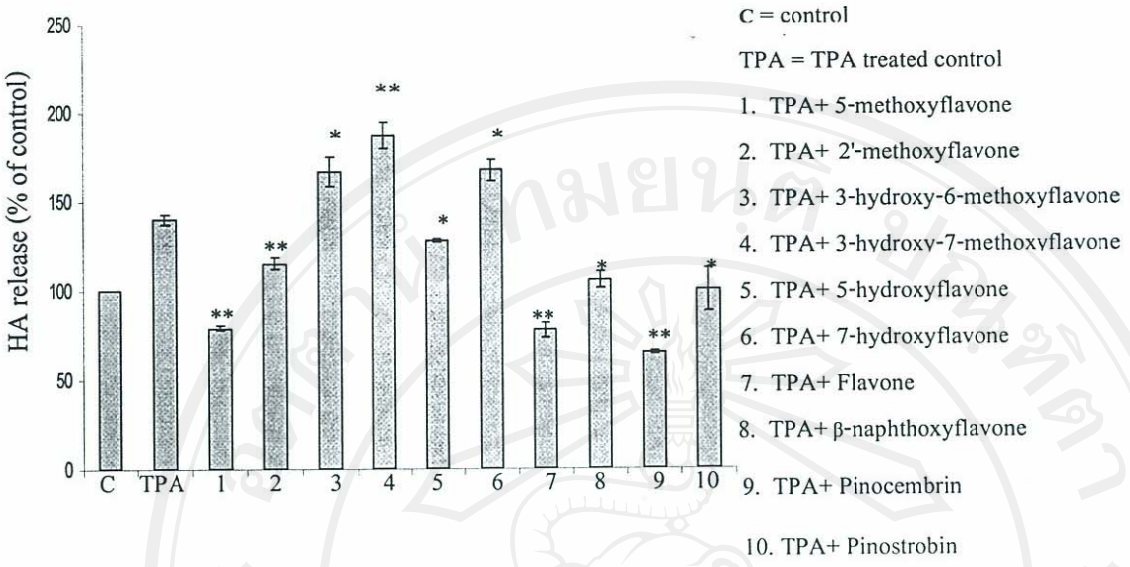



**Figure 17.** Inhibitory effect of pinocembrin, pinostrobin and flavonoid derivatives on the release of HA induced by TPA from oral fibroblast medium. Cells were treated with 100 ng/ml TPA and 10 µg/ml pinocembrin, pinostrobin and flavonoid derivatives, solvent control (DMSO) and 100 ng/ml TPA-treated control .Data shown are mean value ±standard deviation of triplicate assay per treatment. *, ** Denoted values that were significantly different from TPA-treated control, ($p<0.05$) and ($p<0.01$) respectively.

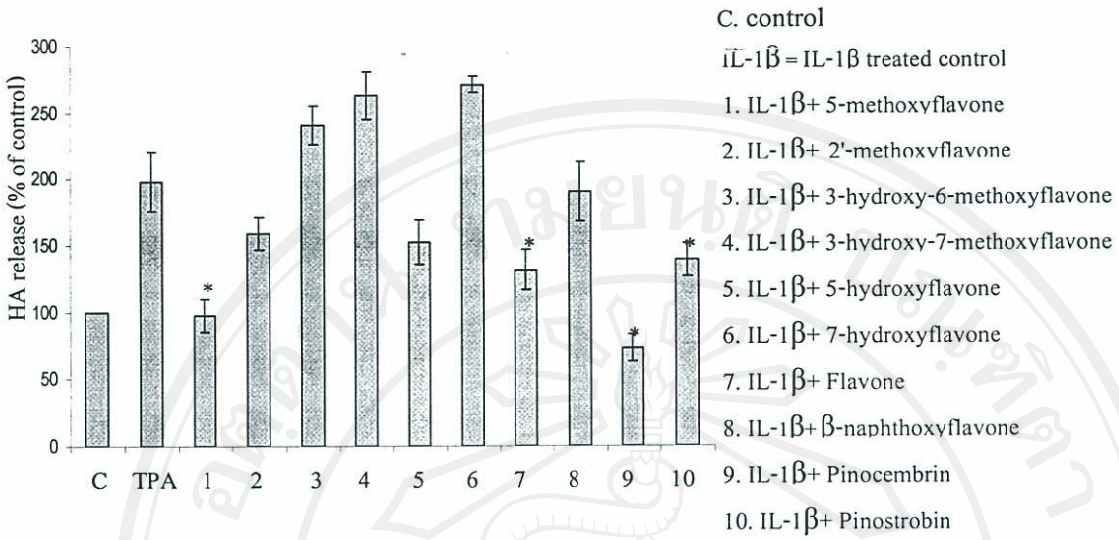



**Figure 18.** Inhibitory effect of pinocembrin, pinostrobin and flavonoid derivatives on the release of HA induced by IL-1β from oral fibroblast medium. Cells were treated with 10 ng/ml IL-1β and 10 μg/ml pinocembrin, pinostrobin and flavonoid derivatives, solvent control (DMSO) and 10 ng/ml IL-1β-treated control. Data shown are mean value ±standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from IL-1β-treated control, ($p<0.05$).

**3.4 Effects of pinocembrin, pinostrobin, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and interleukin-1β (IL-1β) on the mRNA level of hyaluronan synthase (HAS) in oral fibroblast culture.**

This study to assessed the expression of three different hyaluronan synthesis genes, HAS1, HAS2, and HAS3, by measuring their mRNA amounts in cultured human oral fibroblast and investigated the effects of pinocembrin, pinostrobin, TPA and IL-1β

Oral fibroblasts were treated with 100 μg/ml TPA in the presence of pinocembrin and pinostrobin at dose 3, 10, 30μg/ml. After 12 hr, cells were lysed and total RNA were extracted to measured the absolute amounts of HAS mRNA using RT-PCR. The results showed that HAS2 and HAS3 mRNA were detected in oral fibroblast (Figure 19, 20, 21, 22) but HAS1 mRNA could not be detected even at high cycle numbers (Figure 23). Treatment of cells with TPA resulted in moderate and markedly increases the expression of HAS2 (~1.6fold) and HAS3 (~4.6 fold), respectively) (Figure 19 and 20). Pinocembrin markedly decreased the expression of HAS 2 stimulated by TPA and moderate decreased the expression of HAS3 stimulated by TPA at dose 3-30 μg/ml (0.5-1.4 and 0.3-1.4 fold, respectively). Pinostrobin markedly decreased the expression of HAS 2 stimulated by TPA at dose 3-30μg/ml (1.1-1.3 fold ) and moderate decreased the expression of HAS3 stimulated by TPA at dose 30μg/ml ( 1.3 fold ) , (Figure 19 and 20).

To examined the effects of pinocembrin and pinostrobin on IL-1β -induced HA synthase mRNA expression, the mRNA of HAS 2 and HAS 3 were measured at different time (6 h and 24 h respectively) using the same method. The results showed that treatment of cells with IL-1β resulted in marked increases the expression of HAS2 (~11.6 fold) and HAS3 (~ 5.9fold), (Figure 21 and 22). Pinocembrin markedly decreased the expression of HAS 2 and HAS3 stimulated by IL-1β at dose 3-30 μg/ml (6.5-8.5 and 1.5-3.7 folds, respectively). Pinostrobin markedly decreased the expression of HAS 2 and HAS3 stimulated by IL-1β at dose 3-30 μg/ml (4.0-6.3 and 2.3-3.3 fold, respectively) (Figure 21 and 22).

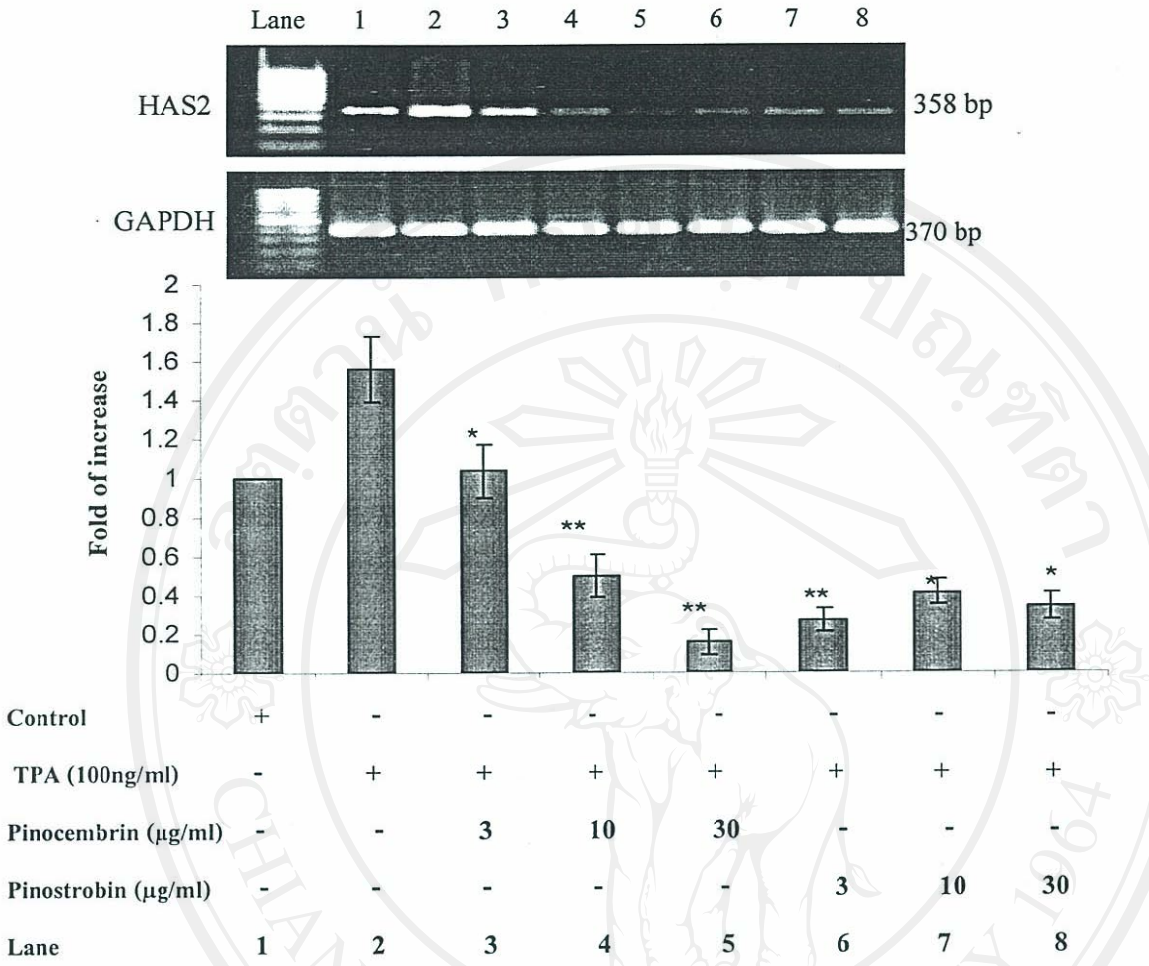



**Figure 19**. Inhibitory effect of various concentrations of pinocembrin and pinostrobin on the mRNA level of HAS 2 induced by TPA in oral fibroblast. Cells were treated with 100 ng/ml TPA and pinocembrin and pinostrobin added at doses 3, 10, 30µg/ml, solvent control (DMSO) and 100 ng/ml TPA-treated control for 12 h .Data shown are mean value ±standard deviation of triplicate assay per treatment. *, ** Denoted values that were significantly different from TPA-treated control, ($p<0.05$) and ($p<0.01$) respectively.

$$\text{Fold of increase} = \frac{\text{Density of sample / Density of GAPDH}}{\text{Density of untreated control / Density of GAPDH}}$$

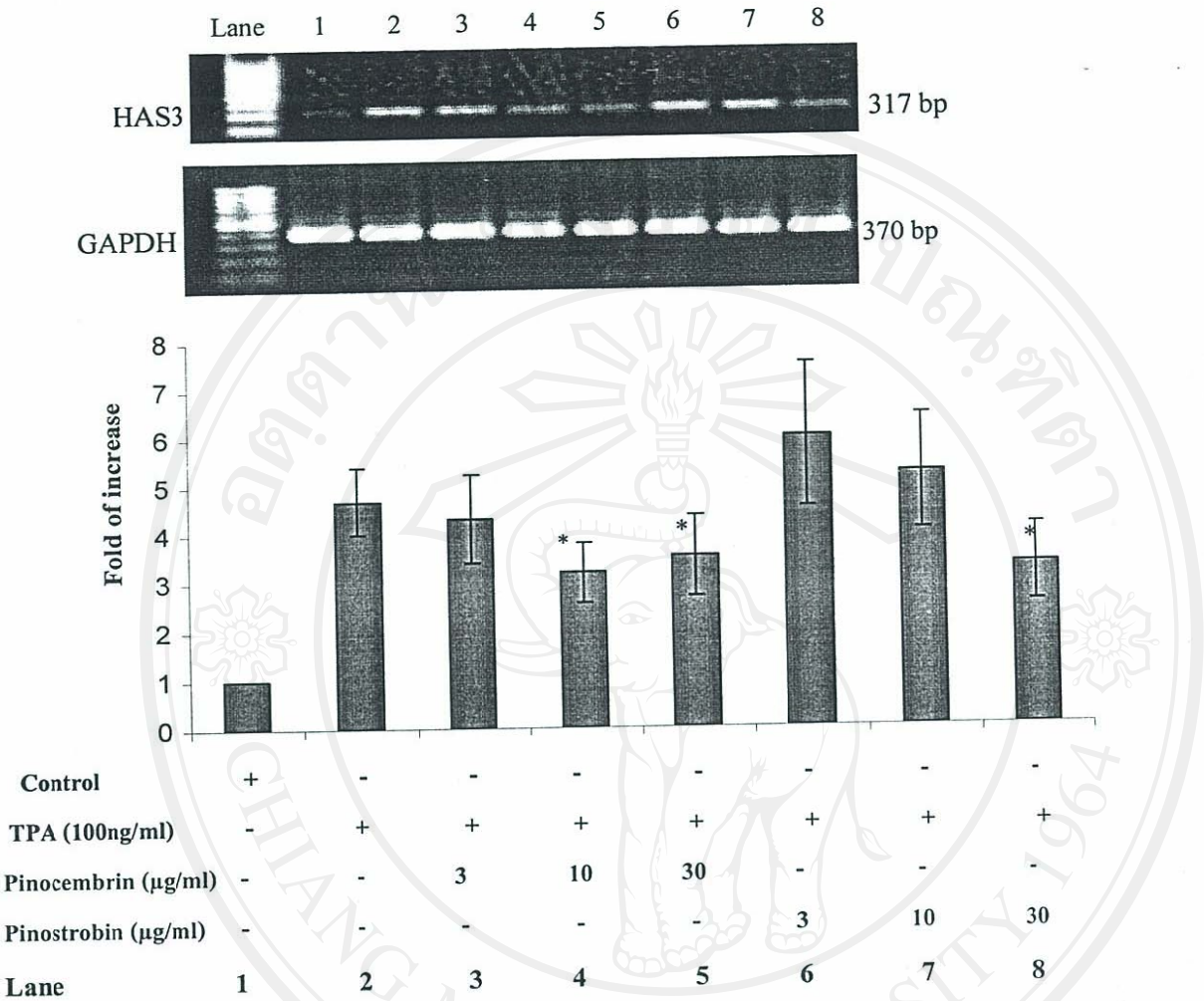



**Figure 20.** Inhibitory effect of various concentrations of pinocembrin and pinostrobin on the mRNA level of HAS3 induced by TPA in oral fibroblast. Cells were treated with 100 ng/ml TPA and pinocembrin and pinostrobin added at doses 3, 10, 30 µg/ml, solvent control (DMSO) and 100 ng/ml TPA-treated control for 12. Data shown are mean value ±standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from TPA-treated control, ($p<0.05$.

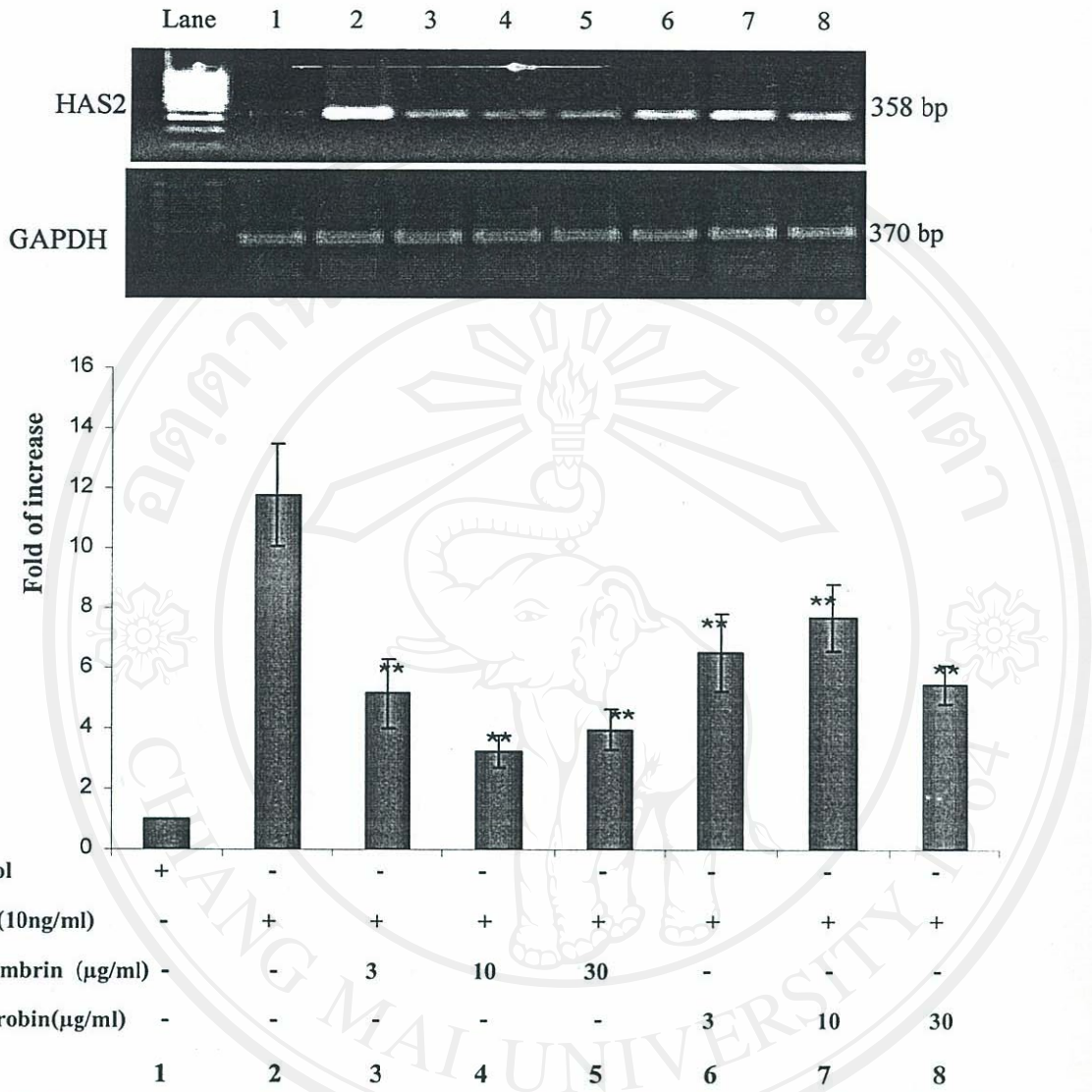



**Figure 21**. Inhibitory effect of various concentrations of pinocembrin and pinostrobin on the mRNA level of HAS 2 induced by IL-1β in oral fibroblast. Cells were treated with 10 ng/ml IL-1β and pinocembrin and pinostrobin added at doses 3, 10, 30µg/ml, solvent control (DMSO) and 10 ng/ml IL-1β-treated control for 6 h .Data shown are mean value ±standard deviation of triplicate assay per treatment. ** Denoted values that were significantly different from IL-1β-treated control, ($p<0.01$).

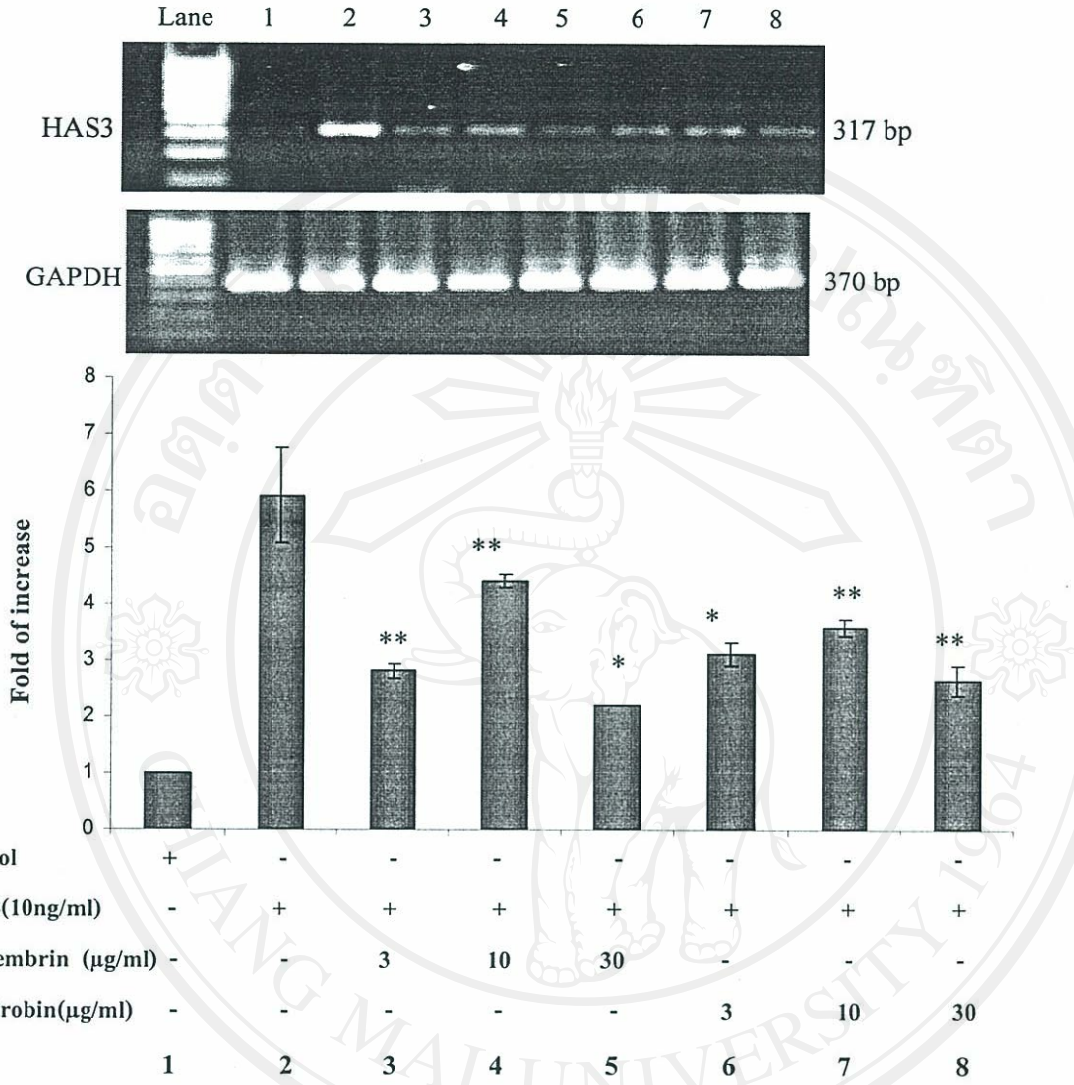
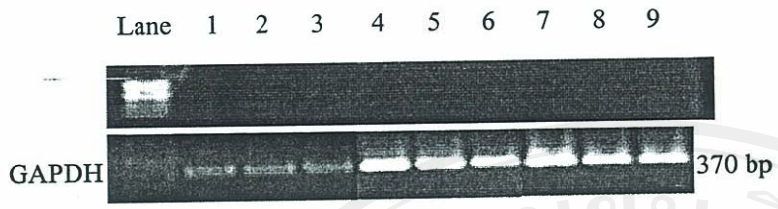



**Figure** 22. Inhibitory effect of various concentrations of pinocembrin and pinostrobin on the mRNA level of HAS 3 induced by IL-1β in oral fibroblast. Cells were treated with 10 ng/ml IL-1β and pinocembrin and pinostrobin added at doses 3, 10, 30µg/ml, solvent control (DMSO) and 10 ng/ml IL-1β-treated control for 24 h .Data shown are mean value ±standard deviation of triplicate assay per treatment. *, ** Denoted values that were significantly different from IL-1β-treated control, ($p<0.05$) and ($p<0.01$) respectively.




1. Control (6 hr.)
2. DMSO (6 hr.)
3. IL-1β (6 hr.)
4. CONTROL (24 hr.)
5. DMSO (24 hr.)
6. IL-1β (24 hr.)
7. CONTROL (12hr.)
8. DMSO (12hr.)
9. TPA (12hr.)

**Figure 23**. Effect of TPA and IL-1ß on the mRNA level of HAS 1 in oral fibroblast. Cells were treated with 10ng/ml IL-1β, 100μg/ml TPA, solvent control (DMSO) and untreated control at different time.

**3.5 Effects of flavonoid derivatives on the mRNA level of hyaluronan synthase (HAS) in oral fibroblast culture.**

To compare the effect of flavonoid compounds on HAS gene expression, oral fibroblast were treated with 100 μg/ml TPA in the presence of flavonoids derivatives, pinocembrin and pinostrobin at dose 10μg/ml. After 12 hr, cells were lysed and total RNA were extracted. Quantization of each mRNA was performed using real-time RT-PCR. The results showed that all flavonoids inhibited both of HAS2 and HAS3 mRNAs expression stimulated by TPA (Figure 24 and 25). Only 5-methoxyflavone was found to inhibit HAS 2 mRNA expression (Figure 24), however, it induced HAS 3 mRNA expression (Figure 25).

To examined the effects of flavonoid compounds at the same concentration on the expression of HAS mRNAs stimulated by IL-1β, the mRNA of HAS2 and HAS3 were measured at different time (6 h and 24 h respectively) using the same method. The results showed that all flavonoids significantly inhibited HAS2 mRNA expression stimulated by IL-1β. β-napthoxyflavone, flavone, pinocembrin and pinostrobin significantly inhibited HAS3 mRNA expression, while 3-hydroxy-6-methoxyflavone and 3-hydroxy-7-methoxyflavone induced increase in HAS3 expression stimulated by IL-1β although it was not significant (Figure 26 and 27).

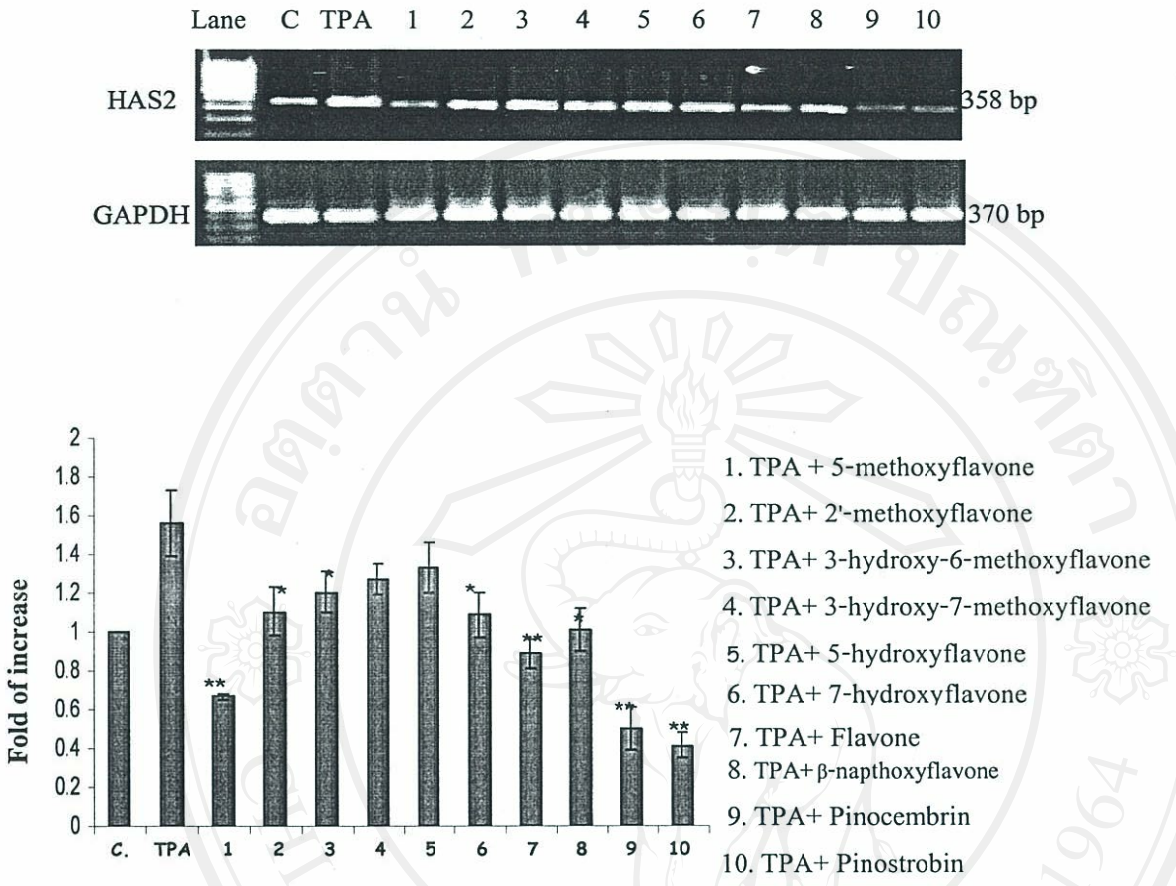



**Figure 24.** Inhibitory effect of pinocembrin, pinostrobin and flavonoid derivatives on the mRNA level of HAS2 induced by TPA in oral fibroblast. Cells were treated with 100 ng/ml TPA and 10 µg/ml, pinocembrin, pinostrobin and flavonoid derivatives, solvent control (DMSO) and 100 ng/ml TPA-treated control .Data shown are mean value ±standard deviation of triplicate assay per treatment. *,** Denoted values that were significantly different from TPA-treated control, ($p<0.05$) and ($p<0.01$) respectively.

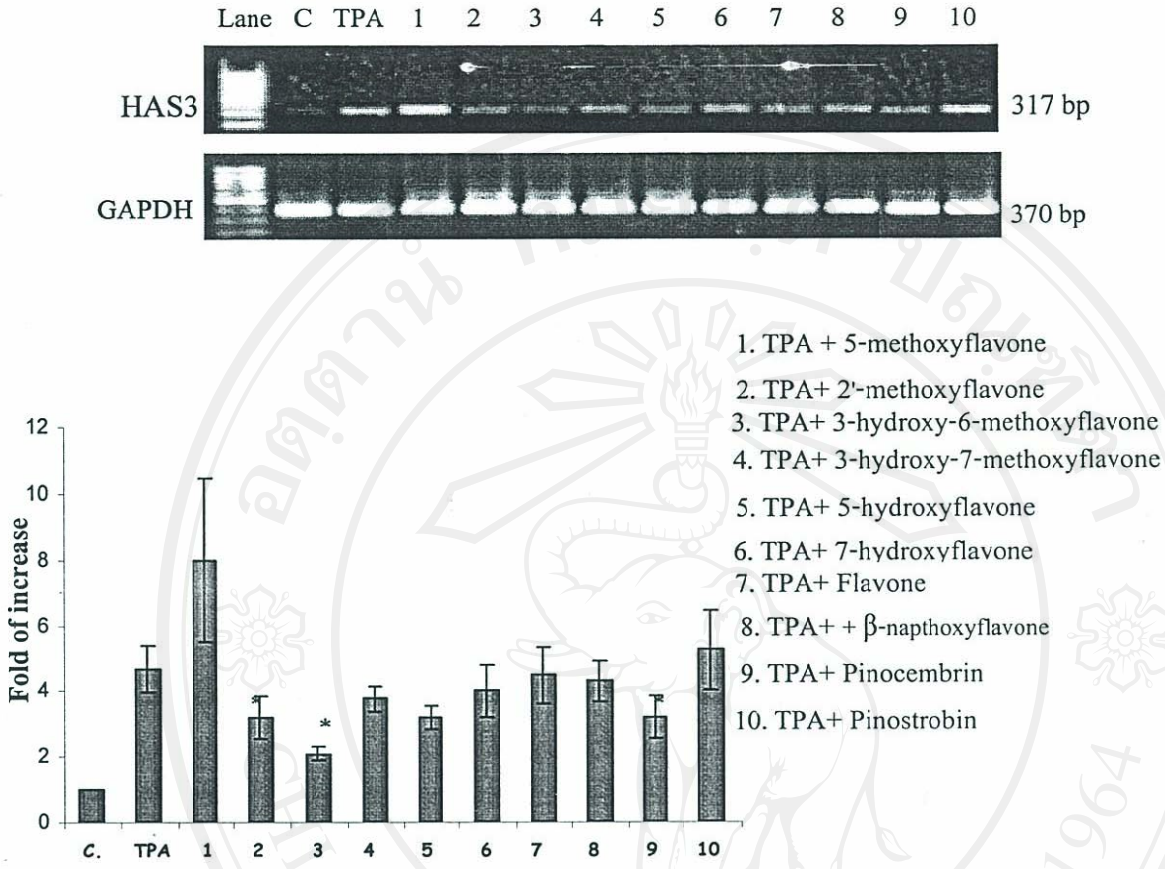



**Figure 25.** Inhibitory effect of pinocembrin, pinostrobin and flavonoid derivatives on the mRNA level of HAS3 induced by TPA in oral fibroblast. Cells were treated with 100 ng/ml TPA and 10 μg/ml pinocembrin, pinostrobin and flavonoid derivatives, solvent control (DMSO) and 100 ng/ml TPA-treated control .Data shown are mean value ±standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from TPA-treated control, ($p<0.05$).

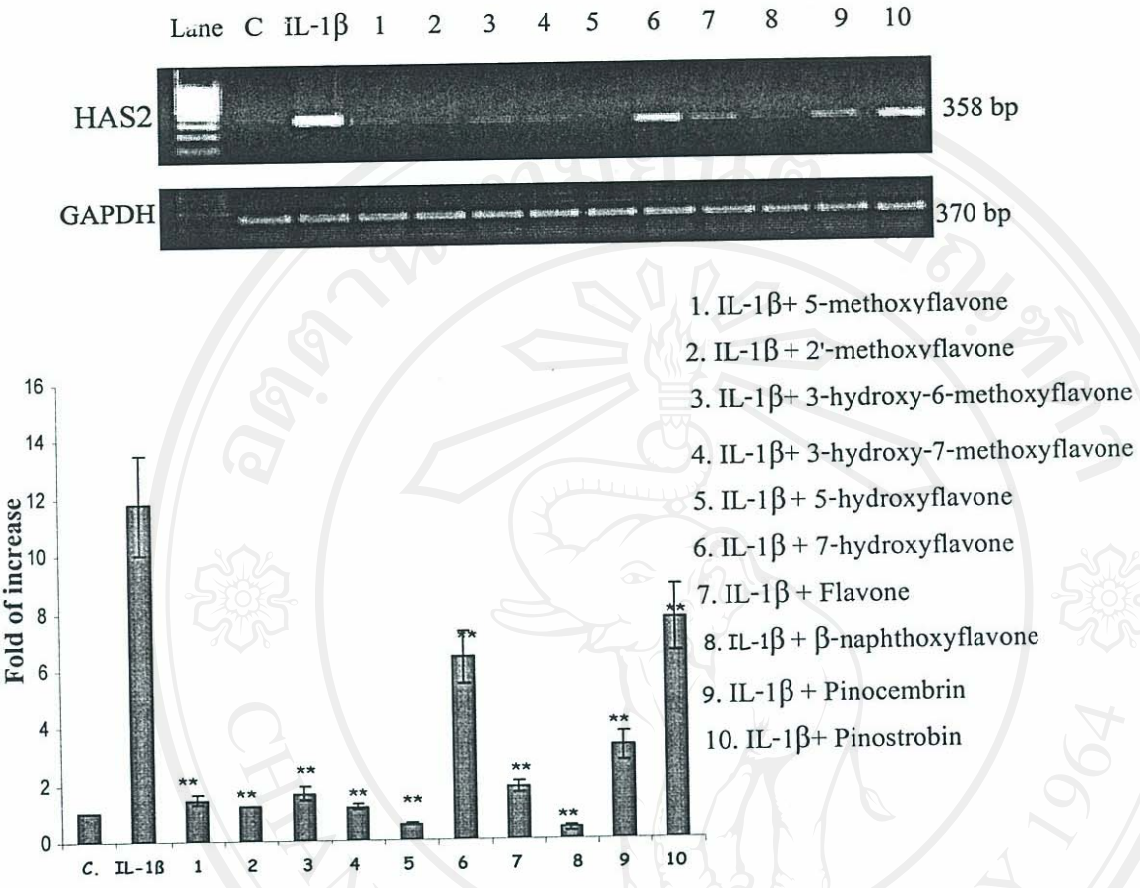



**Figure 26.** Inhibitory effect of pinocembrin, pinostrobin and flavonoid derivatives on the mRNA level of HAS 2 induced by IL-1β in oral fibroblast. Cells were treated with 10 ng/ml IL-1β and 10 μg/ml pinocembrin, pinostrobin and flavonoid derivatives, solvent control (DMSO) and 10 ng/ml IL-1β-treated control .Data shown are mean value ±standard deviation of triplicate assay per treatment. ** Denoted values that were significantly different from IL-1β-treated control, ($p<0.01$).

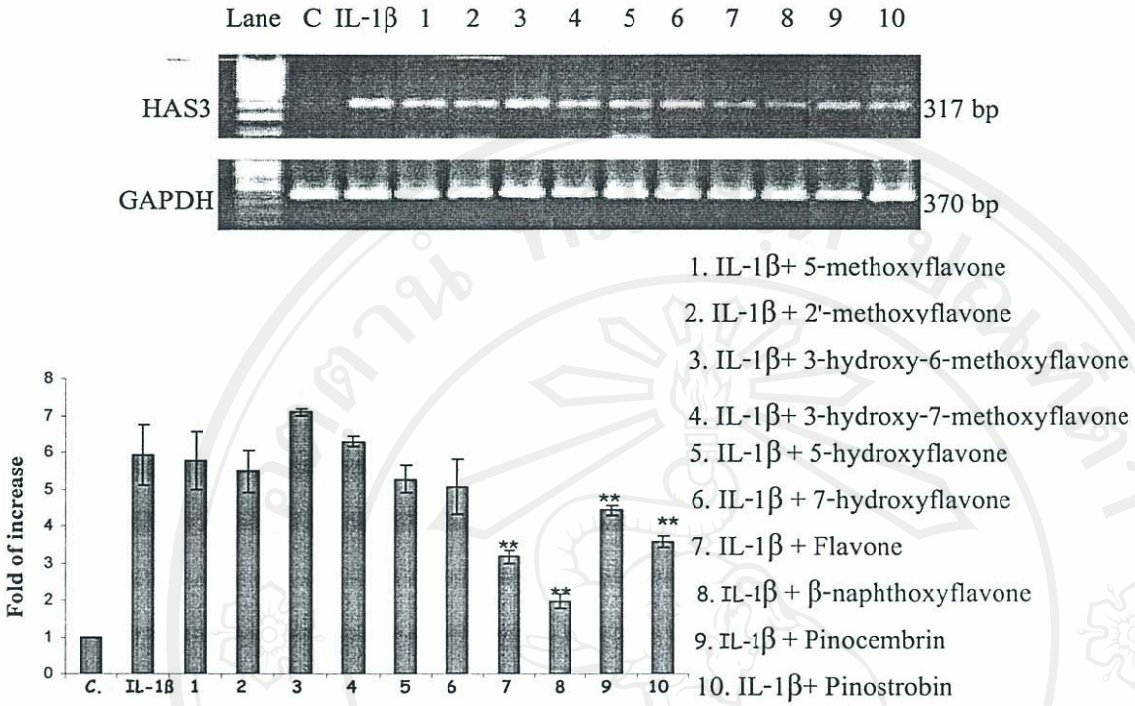



**Figure 27.** Inhibitory effect of pinocembrin, pinostrobin and flavonoid derivatives on the RNA level of HAS 3 induced by IL-1β in oral fibroblast. Cells were treated with 10 ng/ml IL-1β and 10 μg/ml pinocembrin, pinostrobin and flavonoid derivatives, solvent control (DMSO) and 10 ng/ml IL-1β-treated control .Data shown are mean value ±standard deviation of triplicate assay per treatment. ** Denoted values that were significantly different from IL-1β-treated control, ($p<0.01$).

**3.6 Cytotoxic effects of *B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives on oral fibroblast.**

To examined whether B. *pandurata* Extracts, pinocembrin, pinostrobin and flavonoid derivatives affect cytotoxicity of cells, oral fibroblast were exposed to these compounds and cytotoxicity were determined by Berger-Broida assay and MTT assay.

**3.6.1 Cytotoxic effects of *B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives on cytoplasmic enzyme activity releaesed by damaged cells.**

The cytotoxicity of the compound to fibroblasts is base on the measurement of cytoplasmic enzyme activity released by damaged cells. Oral fibroblasts were treated with *B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives and 5.0mM $H_2O_2$ overnight .The culture media were collected to determine the cytotoxicity by Berger-Broida assay. The results showed that the cytoplasmic enzyme activity released in treated culture medium (*B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives) not significant different to the untreated control .The cytoplasmic enzyme activity released was significantly increased by $H_2O_2$ (positive control) as showed in Figure 28.

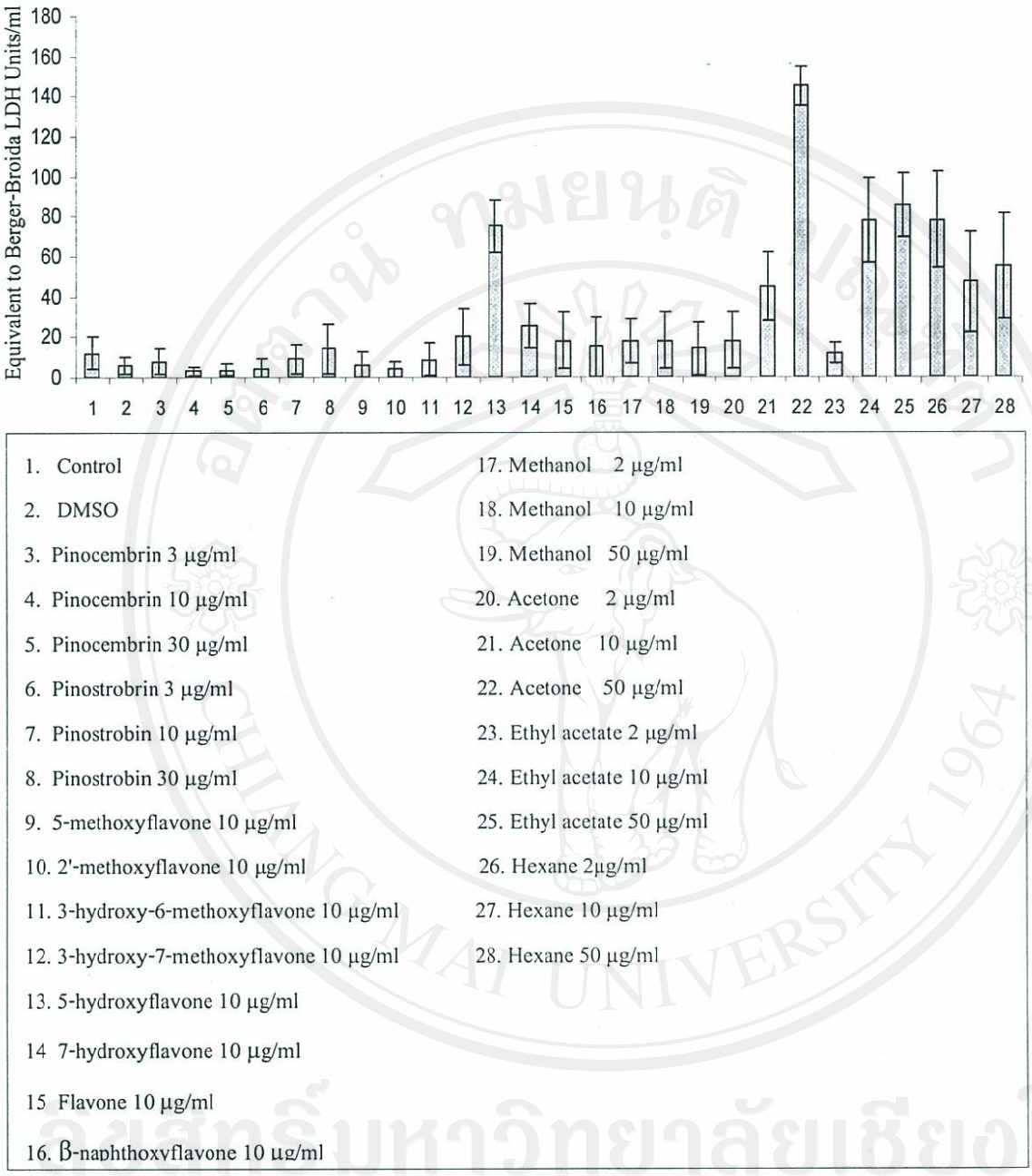



**Figure 28.** Effects of *B. pandurata* extracts on cytoplasmic enzyme activity released by damaged cells. Cells were treated with methanol, acetone, ethyl acetate extract and hexane, pinocembrin, pinostrobin and flavonoid derivatives added at different doses, untreated control, solvent ontrol(DMSO), and 5.0mM $H_2O_2$ (positive control). Data shown are mean value ±standard deviation of triplicate assay per treatment.

**3.6.2 Effects of *B. pandurata.* extracts, pinocembrin, pinostrobin, flavonoid derivatives on the survival of oral fibroblast cells.**

Oral fibroblasts were treated with 10 µg/ml of *B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives and 5.0mM $H_2O_2$ overnight. The culture fibroblasts were collected to determine the cell survival by MTT assay. The results showed that a measure of the viability of cells (*B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives) were not significant different to the untreated control. However, the viability of cells treated with 50 µg/ml of hexane and ethyl acetate extracts were significant different to the untreated control. The dead cells was significantly increased by $H_2O_2$ (positive control) as showed in Figure 29.

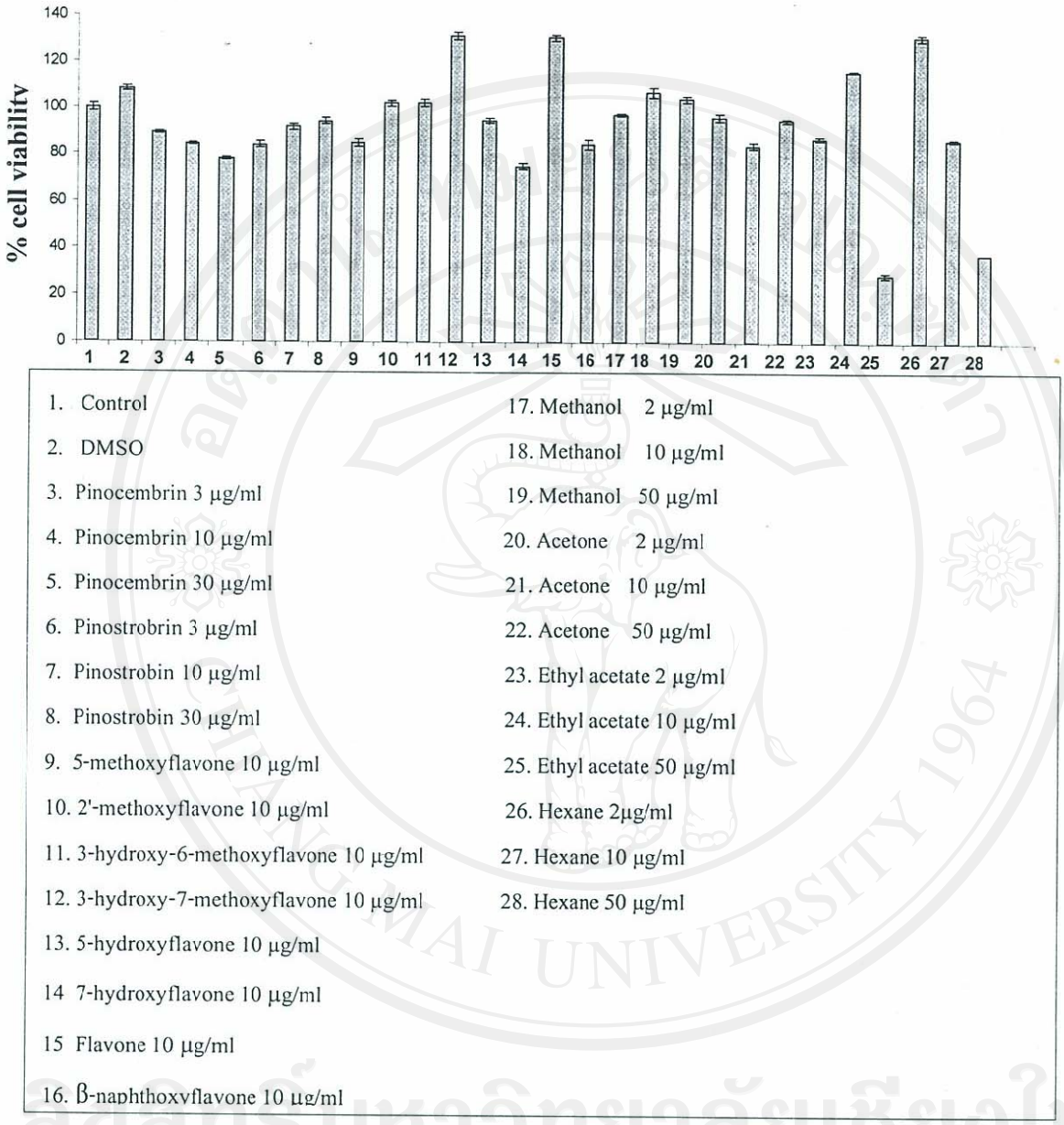
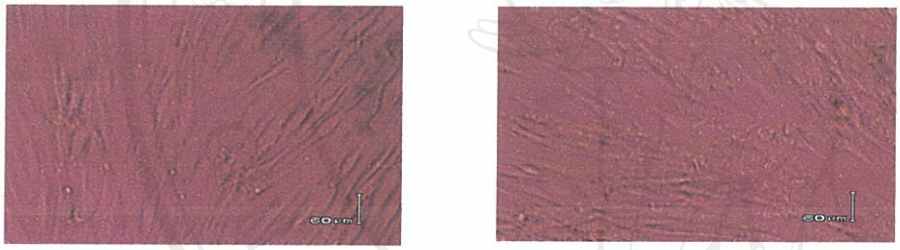



**Figure 29.** Effects of *B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives on cell survival of oral fibroblast. Cells were treated with methanol, acetone, ethyl acetate and hexane extracts, pinocembrin, pinostrobin and flavonoid derivatives added at different doses, untreated control, solvent control (DMSO), and 5.0mM $H_2O_2$ (positive control). Data shown are mean value ± standard deviation of triplicate assay per treatment.

### 3.7 Effect of *B. pandurata* extracts pinocembrin, pinostrobin and flavonoid derivatives on phenotype of oral fibroblast cells.

In this study, *B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives were added to the medium of oral fibroblast. It investigated the morphology of cells by microscopy . The result showed that at all concentrations of *B. pandurata* extracts, pinocembrin, pinostrobin and flavonoid derivatives, but not 50µg/ml ethyl acetate and hexane extracts (the result shown in Figure 11.), did not change phenotype of oral fibroblast as compare to the untreated primary cells. The result of 10 µg/ml pinocembrin shown in Figure 30 but results of all concentrations of other compounds which were similar to 10 µg/ml pinocembrin were not shown.



A. Normal control B. Pinostrobin 10 µg/ml

**Figure 30** Effect of 10 µg/ml pinocembrin on phenotype of oral fibroblast cells. Cells were treated with methanol, hexane, acetone and ethyl acetate extracts , pinocembrin, pinostrobin and flavonoid derivatives added at different doses, untreated control, solvent control (DMSO), and 5.0mM $H_2O_2$ (positive control).