CHAPTER III

RESULTS

3.1 Effect of *Zingiber cassumunar* Roxb. extract on phenotype of oral fibroblast and epithelial cells

The rhizome of *Zingiber cassumunar* Roxb. (Plai) is widely used in Thai traditional medicine for topical treatment of joint inflammations, muscular pain, and similar inflammation-related disorders. In this study, three extract fractions were added to the medium of oral fibroblast and epithelial cells. Treatment of the extract for 24 hours, we investigated the morphology of both cells by microscopy. The result showed that *Zingiber cassumunar* Roxb extract did not change phenotype of both cells type as compare to untreated primary cells, the result shown in Figure 10.

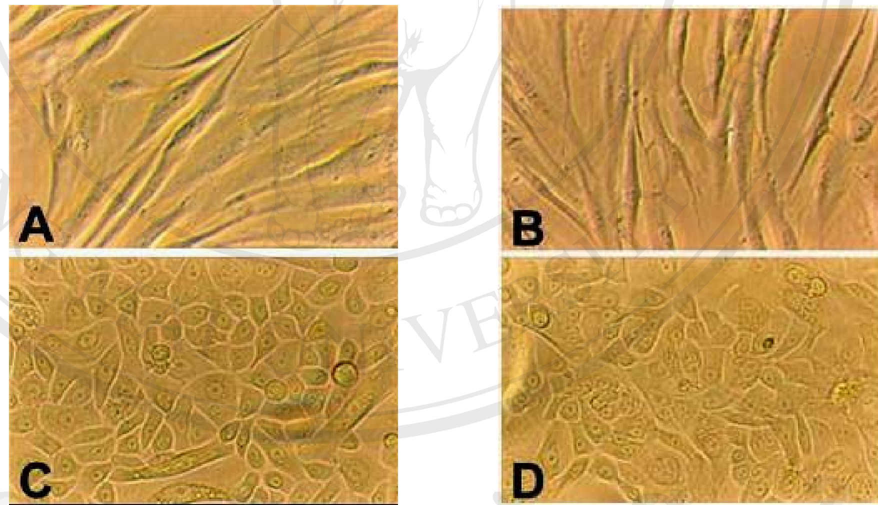

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Figure 10. Inhibitory effect of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the releases of HA in oral fibroblast medium. Cells were treated with ethanol, hexane, and water extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml and control (untreated). Data are the mean values ± standard deviation of triplicate per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

3.2 Effects of *Zingiber cassumunar* Roxb. extract on the level of extracellular matrix (ECM); HA, sulfated-GAG, MMP-2, -9, and the cytotoxicity from oral cells cultured medium.

The rhizome of *Zingiber cassumunar* Roxb. (Plai) is widely used in Thai traditional medicine for topical treatment of joint inflammations, muscular pain, and similar inflammation-related disorders. Preliminary studies revealed the effect of (E)-1-(3-4-dimethoxyphynyl) butadiene (DMPBD) on anti-inflammatory activity by action on both cyclooxygenase (COX) and lipoxygenase (LOX) in arachidonic acid (AA) metabolic pathways. To study the effect of this extract on the level of HA, sulfated-GAG, MMP-2, 9 and the cytotoxicity, oral fibroblast and epithelial cells were treated with various concentrations of three extract fractions, ethanol, hexane, and water, overnight.

3.2.1 Effects of *Zingiber cassumunar* Roxb. extract, Retinoic acid (RA), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of hyaluronic acid (HA) in oral fibroblast medium.

In this study, three extract fractions were added to the medium of oral fibroblasts. After 24 hours of treatment, the release of HA was investigated in the culture medium by ELISA assay. The results showed that the ethanol and hexane fractions were able to inhibit the release of HA into the culture media in dose dependent manner but not the water fraction. When compare between hexane and ethanol extracts, it was found that the hexane extract of Plai showed less HA in culture media than ethanol extract at the same concentration as shown in Table 3 and Figure 11.

Retinoic acid (RA) was used as inhibitor to inhibit hyaluronate synthesis *in vitro* model. RA was treated at doses 0.1, 1.0, 10.0, and 50.0 μM in the culture medium overnight. The culture media were collected for analysis. The results showed that the release of HA was partially decreased less than untreated control as shown in figure 12. In addition, oral fibroblasts were treated with the combination between 10.0 μM RA and ethanol fraction at doses 0, 0.1, 1.0, 6.3, 12.5, 25.0, and 50.0 μg/ml overnight. The release of HA was decreased as treated only with ethanol extract as showed in figure 13.

Table 3: Inhibitory effect of Plai extracts on the release of HA in oral fibroblast medium

Treatment	Relative change of HA release (%)
Untreated control	100 ± 0
0.1 µg/ml P-EtOH	128.43 ± 28.22
1.0 µg/ml P-EtOH	113.83 ± 43.55
6.3 µg/ml P-EtOH	88.82 ± 26.08
12.5 µg/ml P-EtOH	100.33 ± 18.00
25.0 µg/ml P-EtOH	67.05 ± 29.03*
50.0 µg/ml P-EtOH	59.40 ± 29.87*
0.1 µg/ml P-Hex	138.73 ± 11.47
1.0 µg/ml P-Hex	107.02 ± 9.75
6.3 µg/ml P-Hex	105.49 ± 75.73
12.5 µg/ml P-Hex	74.56 ± 59.43
25.0 µg/ml P-Hex	47.60 ± 37.67*
50.0 µg/ml P-Hex	31.51 ± 13.35*
0.1 µg/ml P-H_2O	140.00 ± 26.83
1.0 µg/ml P- H_2O	151.98 ± 45.55
6.3 µg/ml P- H_2O	141.24 ± 30.97
12.5 µg/ml P- H_2O	162.35 ± 20.21
25.0 µg/ml P- H_2O	152.22 ± 30.03
50.0 µg/ml P- H_2O	178.99 ± 33.03

Data shown are mean value ± standard deviation of triplicate assay per treatment.

* Denoted value that was significantly different from the untreated control ($p<0.05$).

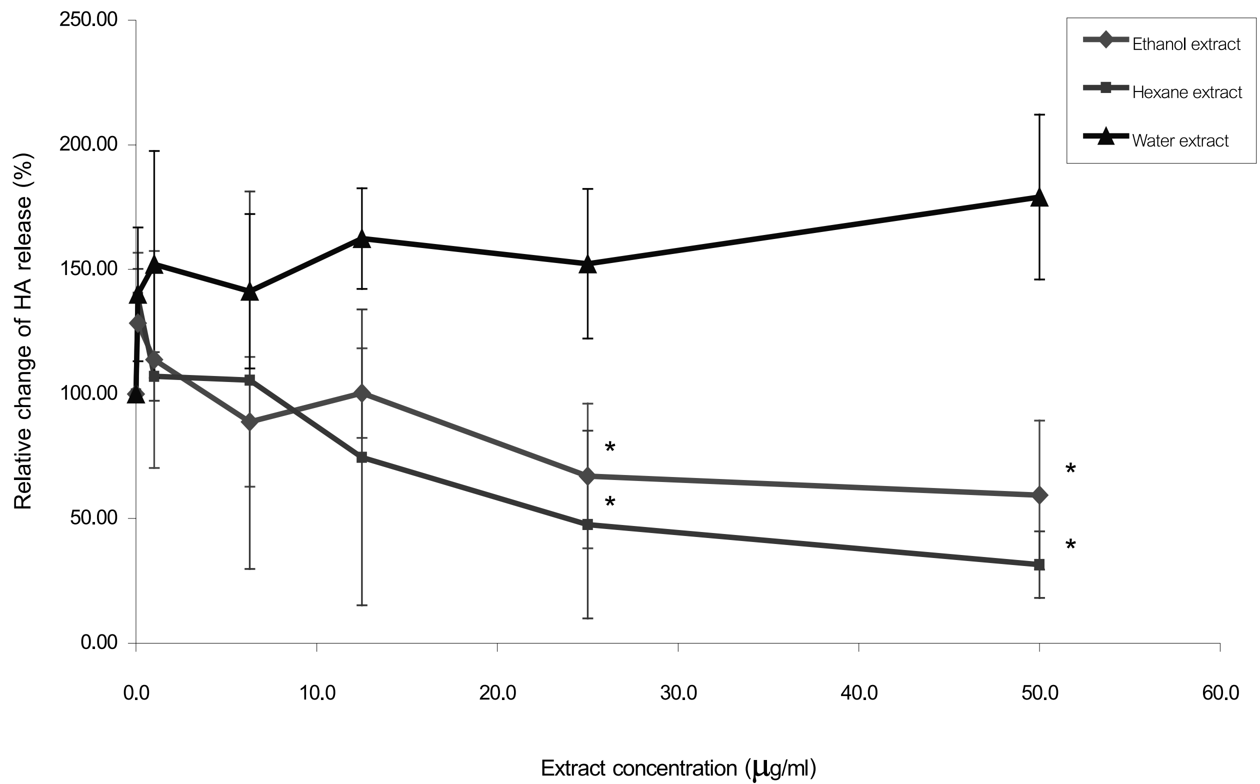

Figure 11. Inhibitory effect of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the releases of HA in oral fibroblast medium. Cells were treated with ethanol, hexane, and water extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml and control (untreated). Data are the mean values ± standard deviation of triplicate per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

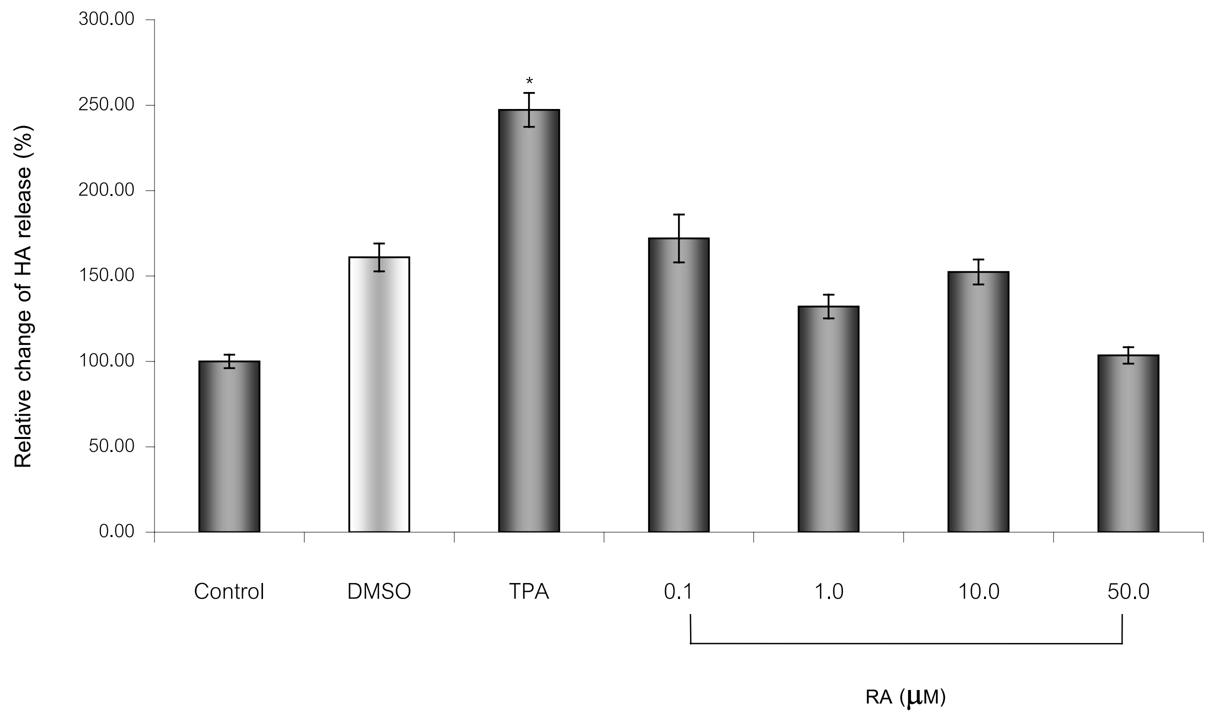

Figure 12. Effect of various concentrations of Retinoic acid (RA) on the releases of HA in oral fibroblast medium. Cells were treated at doses 0.1, 1, 10, 50 μM, control (untreated), solvent control (treated with DMSO) and 1 μg/ml TPA. Data shown are mean value $\pm$ standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

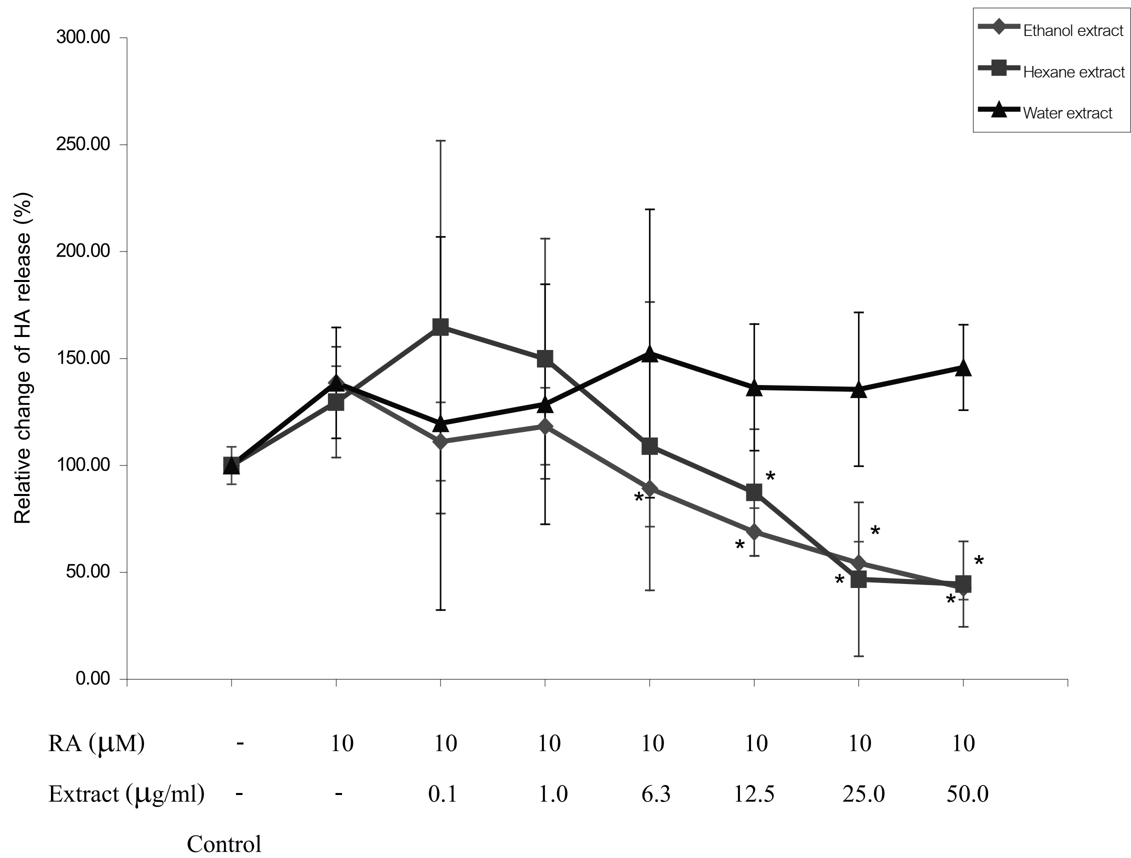

Figure 13. Inhibitory effect of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the release of HA in oral fibroblast medium. Cells were treated with 10 μM RA and the extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml, control (untreated), solvent control (treated with DMSO) and 10 μM RA-treated control. Data shown are mean value $\pm$ standard deviation of triplicate assay per treatment. *Denoted values that were significantly different from RA-treated control (+RA/-Plai), ($p<0.05$).

3.2.2 Effects of *Zingiber cassumunar* Roxb. extract, Retinoic acid (RA), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of HA from oral epithelial medium.

To study the effect of this extract on the level of HA, sulfated-GAG, MMP-2, 9 and the cytotoxicity, oral epithelia were treated with various concentrations of three extract fractions, which are ethanol, hexane and water, overnight.

In this study, the three extract fractions were added to the medium of oral epithelia. The release of HA was investigated in the culture medium by ELISA assay. The result showed that Plai extract from ethanol and hexane fractions were able to stimulate the release of HA into the culture media in the dose dependent manner but the water fraction was not. When compare between hexane and ethanol extracts, it was found that hexane extract of Plai showed more HA in culture media than ethanol extract at the same concentration as shown in Table 4. and Figure 14.

Retinoic acid (RA) at dose 1.0, 10.0, 25.0, 50.0 μM and 1 μg/ml TPA were added to the culture medium overnight. The culture media were collected for analysis. The release of HA seemed to be more than untreated control at doses 1.0 and 10.0 μM RA but not in treatment of TPA changed as shown in Figure 15. In addition, oral fibroblasts were treated with the combination of 10.0 μM RA and Plai extract at doses 0, 0.1, 1.0, 6.3, 12.5, 25.0, and 50.0 μg/ml overnight. The results showed that the release of HA was increased 2 fold as compare with treatment only Plai extract as shown in Figure 16.

Table 4: Inhibitory effects of Plai extracts on the release of HA in oral epithelial medium

Treatment	Relative change of HA release (%)
Untreated control	100 ± 0
0.1 μg/ml P-EtOH	90.44 ± 11.26
1.0 μg/ml P-EtOH	81.23 ± 15.38
6.3 μg/ml P-EtOH	160.66 ± 71.57
12.5 μg/ml P-EtOH	168.14 ± 36.57
25.0 μg/ml P-EtOH	264.86 ± 74.39*
50.0 μg/ml P-EtOH	312.49 ± 28.00*
0.1 μg/ml P-Hex	74.35 ± 11.47
1.0 μg/ml P-Hex	98.87 ± 19.75
6.3 μg/ml P-Hex	193.13 ± 65.73
12.5 μg/ml P-Hex	265.45 ± 32.43
25.0 μg/ml P-Hex	299.51 ± 74.67*
50.0 μg/ml P-Hex	352.84 ± 31.35*
0.1 μg/ml P-H_2O	74.35 ± 14.83
1.0 μg/ml P- H_2O	108.87 ± 15.55
6.3 μg/ml P- H_2O	83.13 ± 69.97
12.5 μg/ml P- H_2O	115.45 ± 35.23
25.0 μg/ml P- H_2O	99.51 ± 75.03
50.0 μg/ml P- H_2O	112.84 ± 29.03

Data shown are mean value ± standard deviation of triplicate assay per treatment.

* Denoted value that was significantly different from the untreated control ($p<0.05$).

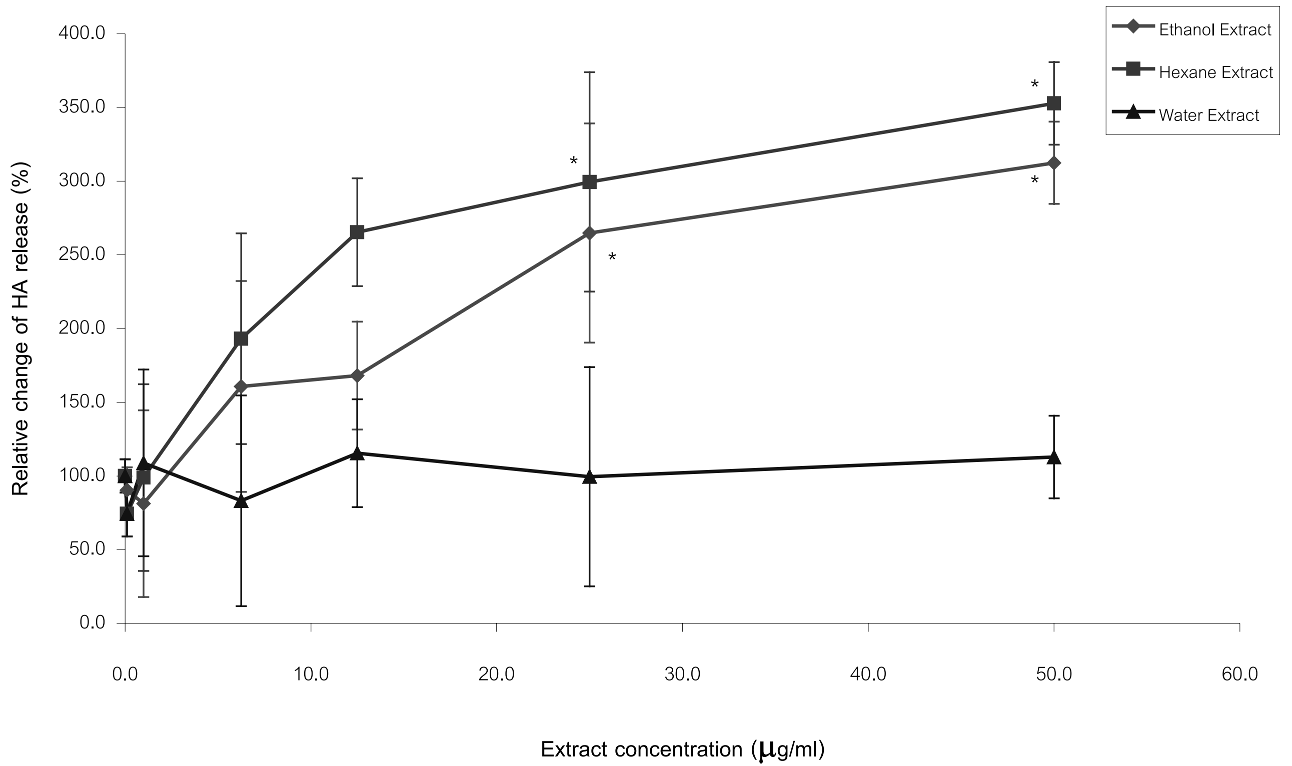

Figure 14. Stimulatory effects of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the release of HA in oral epithelial medium. Cells were treated with ethanol, hexane, and water extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml and control (untreated). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

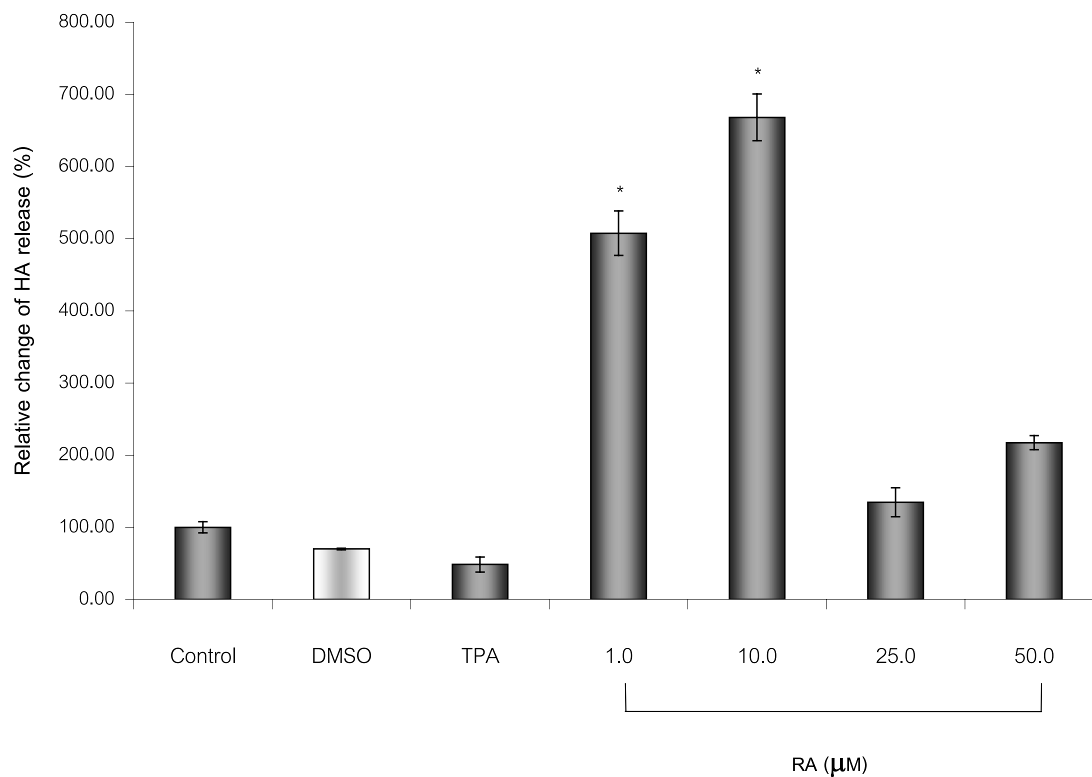

Figure 15. Effect of various concentrations of Retinoic acid (RA) on the release of HA in oral epithelial medium. Cells were treated at doses 1, 10, 25, 50 μM, control (untreated), solvent control (treated with DMSO) and 1 μg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

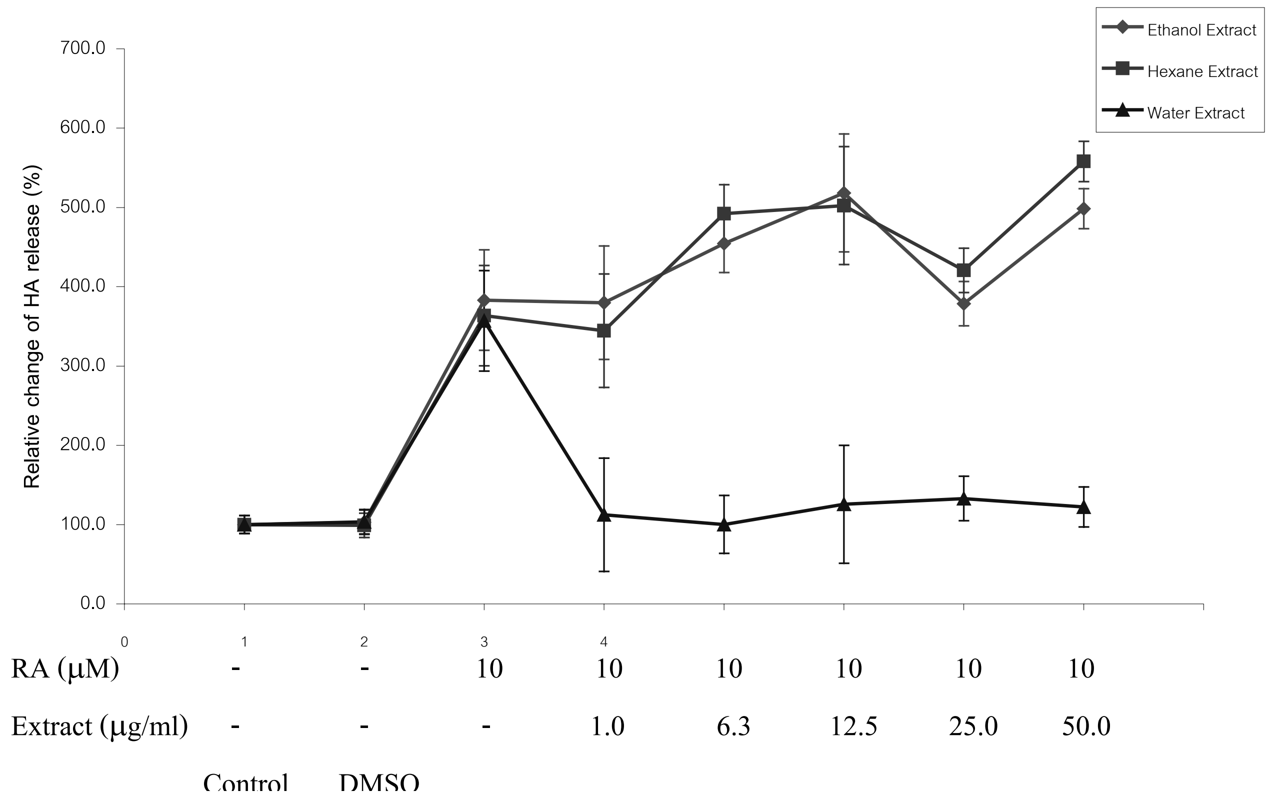

Figure 16. Stimulatory effects of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the releases of HA in oral epithelial medium. Cells were treated with 10 μM RA and the extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml, control (untreated), solvent control (treated with DMSO) and 10 μM RA-treated control**.** Data shown are mean value $\pm$ standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

3.2.3 Effect of *Zingiber cassumunar* Roxb. Extract and Retinoic acid (RA), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of S-GAG from oral fibroblast medium.

Oral fibroblasts were treated with RA at doses 0.1, 1.0, 10.0, 50.0 μM, ethanol extracted Plai at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, combination of 10.0 μM RA and Plai extract at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, and 1.0 μg/ml TPA overnight. The culture media were collected to determine S-GAG content by Farndale reaction assay (dye binding assay). The results showed that the releases of sulfated-GAG was inhibited by RA at doses 0.1-10.0 μM in dose dependent manner as by Plai extract at doses 0.1-25.0 μg/ml as showed in Table 5. and Figure 17. The high concentration of RA (50.0 μM), Plai extract (50.0 μg/ml) and 1 μg/ml TPA were not changed compared with untreated control.

Table 5: Effect of ethanol extracted Plai, RA and TPA on the release of S-GAG in oral fibroblast medium

Treatment	Relative change of S-GAG release (%)
Untreated control	100 ± 3.48
DMSO	159.07 ± 7.34
TPA	113.60 ± 3.83
0.1 µM RA	106.02 ± 2.94
1.0 µM RA	76.35 ± 2.67
10.0 µM RA	66.51 ± 2.23
50.0 µM RA	102.43 ± 3.51
0.1 µg/ml extract	101.64 ± 4.33
1.0 µg/ml extract	72.68 ± 1.55
6.3 µg/ml extract	68.79 ± 1.74
12.5 µg/ml extract	65.79 ± 2.26
25.0 µg/ml extract	59.63 ± 2.28
50.0 µg/ml extract	74.03 ± 2.05
10.0 µM RA + 0.1 µg/ml extract	87.68 ± 3.00
10.0 µM RA + 1.0 µg/ml extract	97.32 ± 4.52
10.0 µM RA + 6.3 µg/ml extract	67.70 ± 3.04
10.0 µM RA + 12.5 µg/ml extract	60.06 ± 2.07
10.0 µM RA + 25.0 µg/ml extract	57.29 ± 1.68
10.0 µM RA + 50.0 µg/ml extract	76.52 ± 1.48

Data shown are mean value ± standard deviation of triplicate assay per treatment.

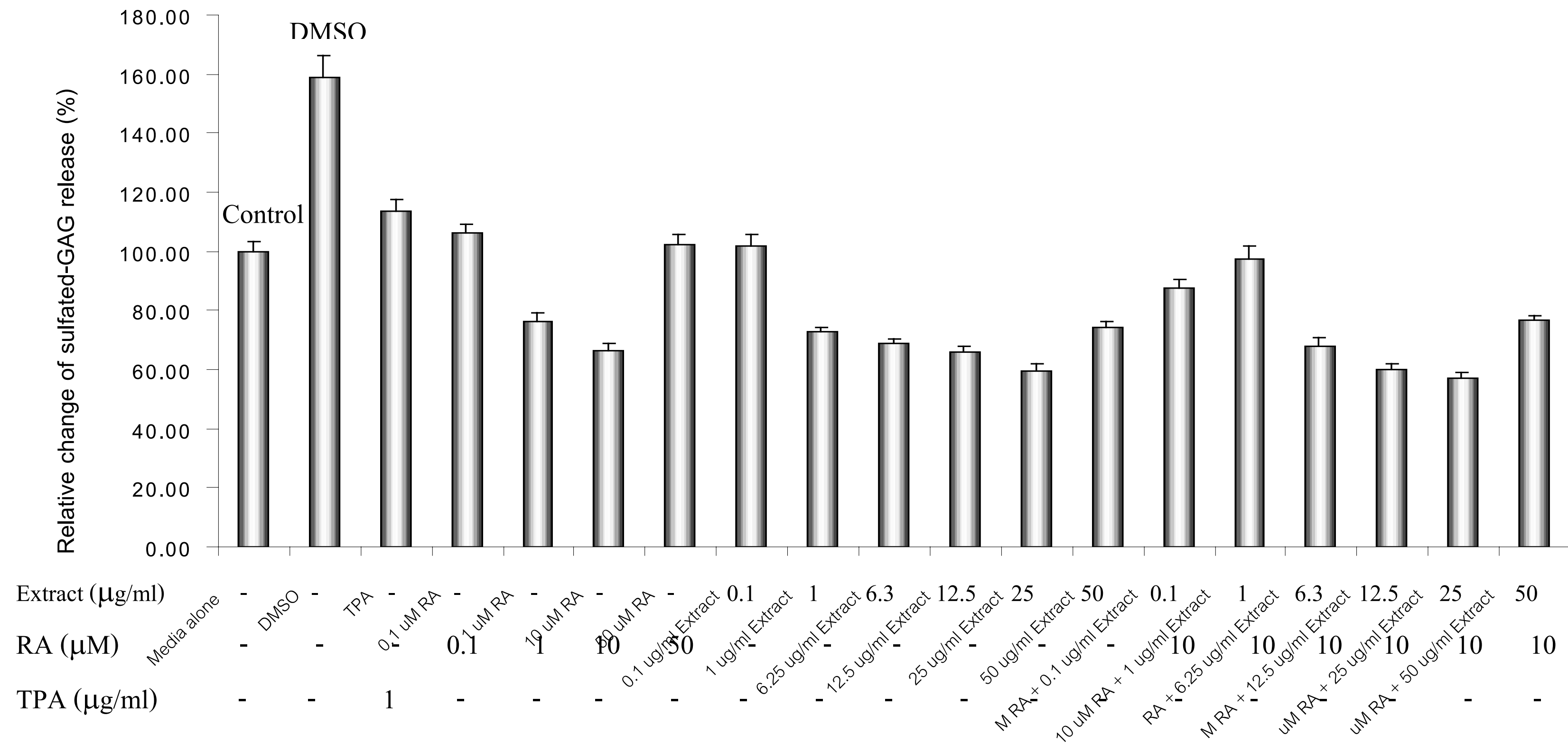

Figure 17. Effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on the releases of S-GAG from oral fibroblast medium. Cells were treated with 10 μM RA and the extract at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml, control (untreated), solvent control (treated with DMSO), 0.1, 1, 10, 50 μM RA, and 1 μg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment.

3.2.4 Effects of *Zingiber cassumunar* Roxb. extract and Retinoic acid (RA), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of S-GAG from oral epithelial medium.

Oral epithelial cells were treated with RA at doses 0.1, 1.0, 10.0, 50.0 μM, ethanol extracted Plai at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, combination of 10.0 μM RA and ethanol extract at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, and 1.0 μg/ml TPA overnight. The culture media were collected to determine S-GAG content by Farndale reaction assay. The results showed that the release of S-GAG was not changed by RA, Plai extract and TPA as shown in Table 6. and Figure 18.

Table 6: Effects of ethanol extracted Plai, RA and TPA on the release of S-GAG in oral epithelial medium

Treatment	Relative change of S-GAG release (%)
Untreated control	100 ± 0.74
DMSO	116.25 ± 0.27
1.0 μM RA	129.60 ± 1.02
10.0 μM RA	102.96 ± 1.16
25.0 μM RA	95.97 ± 1.46
50.0 μM RA	109.69 ± 1.00
1.0 μg/ml extract	83.23 ± 1.02
6.3 μg/ml extract	110.69 ± 2.76
12.5 μg/ml extract	140.01 ± 2.83
25.0 μg/ml extract	142.96 ± 1.93
50.0 μg/ml extract	132.84 ± 0.74
100.0 μg/ml extract	137.58 ± 1.34
10.0 μM RA + 1.0 μg/ml extract	95.29 ± 0.85
10.0 μM RA + 6.3 μg/ml extract	99.87 ± 0.31
10.0 μM RA + 12.5 μg/ml extract	107.03 ± 1.49
10.0 μM RA + 25.0 μg/ml extract	109.69 ± 2.78
10.0 μM RA + 50.0 μg/ml extract	100.00 ± 1.80
10.0 μM RA + 100.0 μg/ml extract	139.10 ± 0.37

Data shown are mean value ± standard deviation of triplicate assay per treatment.

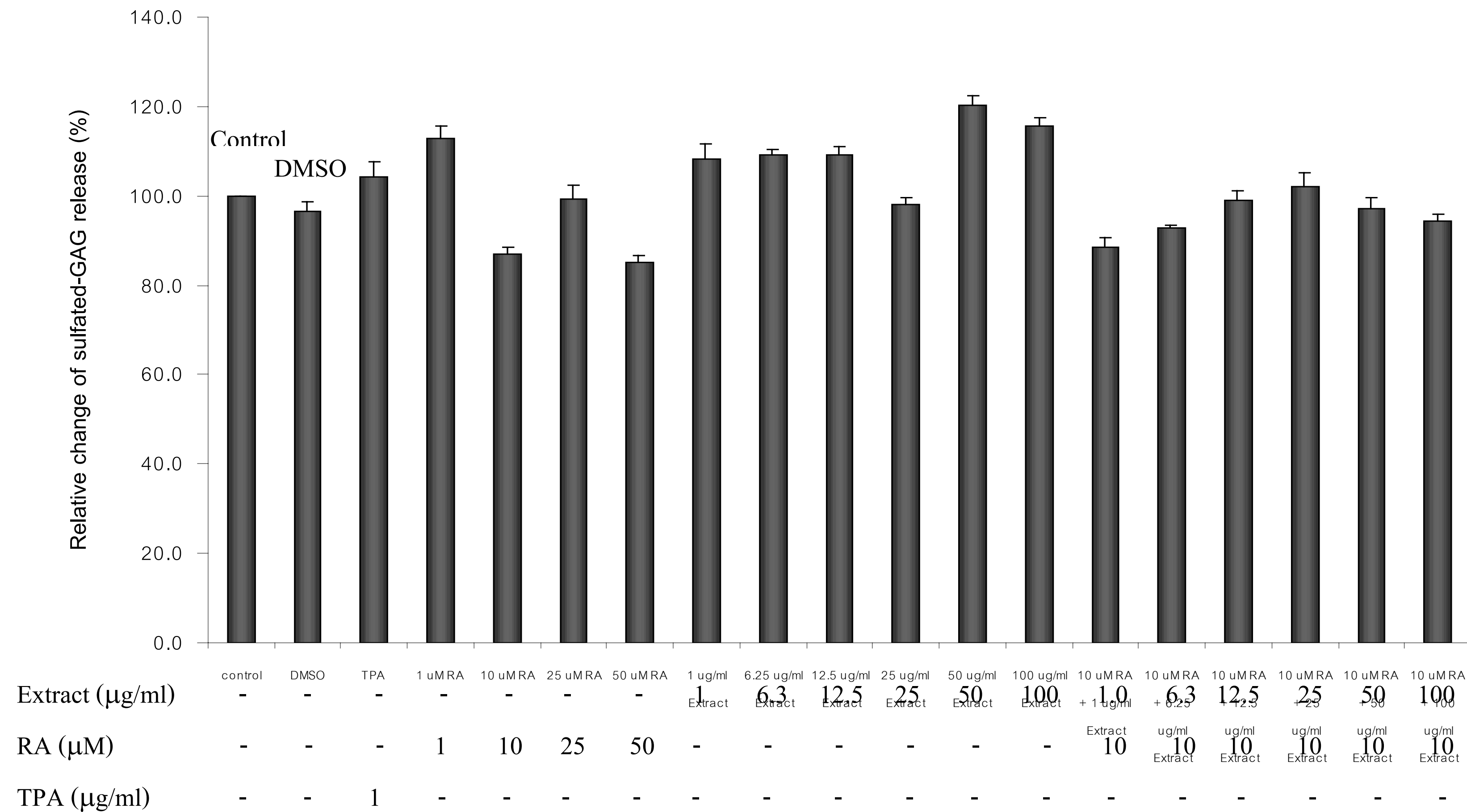

Figure 18. Effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on the releases of sulfated-GAG from oral epithelial medium. Cells were treated with 10 µM RA and the extract at doses 1, 6.25, 12.5, 25, 50, 100 µg/ml, control (untreated), solvent control (treated with DMSO), 0.1, 1, 10, 50 µM RA, and 1 µg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment.

3.2.5 Effect of *Zingiber cassumunar* Roxb. extract, Retinoic acid (RA), and Interleukin-1β (IL-1β) on the level of MMP-2, 9 from oral fibroblast medium.

In this study, activities of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in the culture media were investigated by gelatin zymography. Culture media were electrophoresed on native, non-reducing, gelatin containing gels, which were subsequently stained with Coomassie blue; resolved gelatinolytic proteins were detected as unstained band. Oral fibroblast culture in serum-free medium secreted high levels of gelatinase, which are pro-MMP-2 and pro-MMP-9, when compared to standard enzyme and marker at 72 kDa and 92 kDa respectively.

The results showed that ethanol extracted Plai did not down regulate the production of MMP-2 in the oral fibroblast as compare with untreated control as did the treated-RA, TPA and IL-1β, as shown in Figures 19-21.

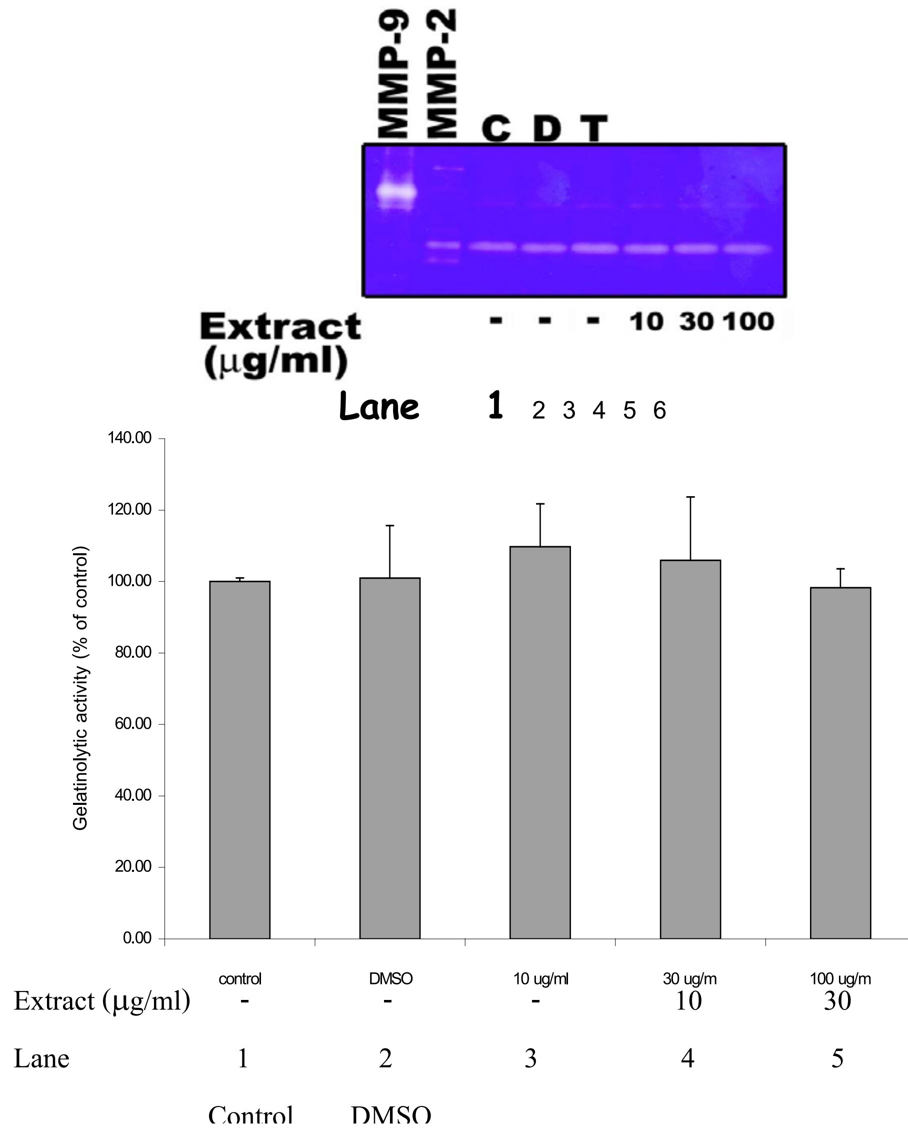

Figure 19. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral fibroblast medium. Cells were treated with the extract was added at doses 10, 30, 100 μg/ml, control (untreated), solvent control (treated with DMSO), and 1 μg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment.

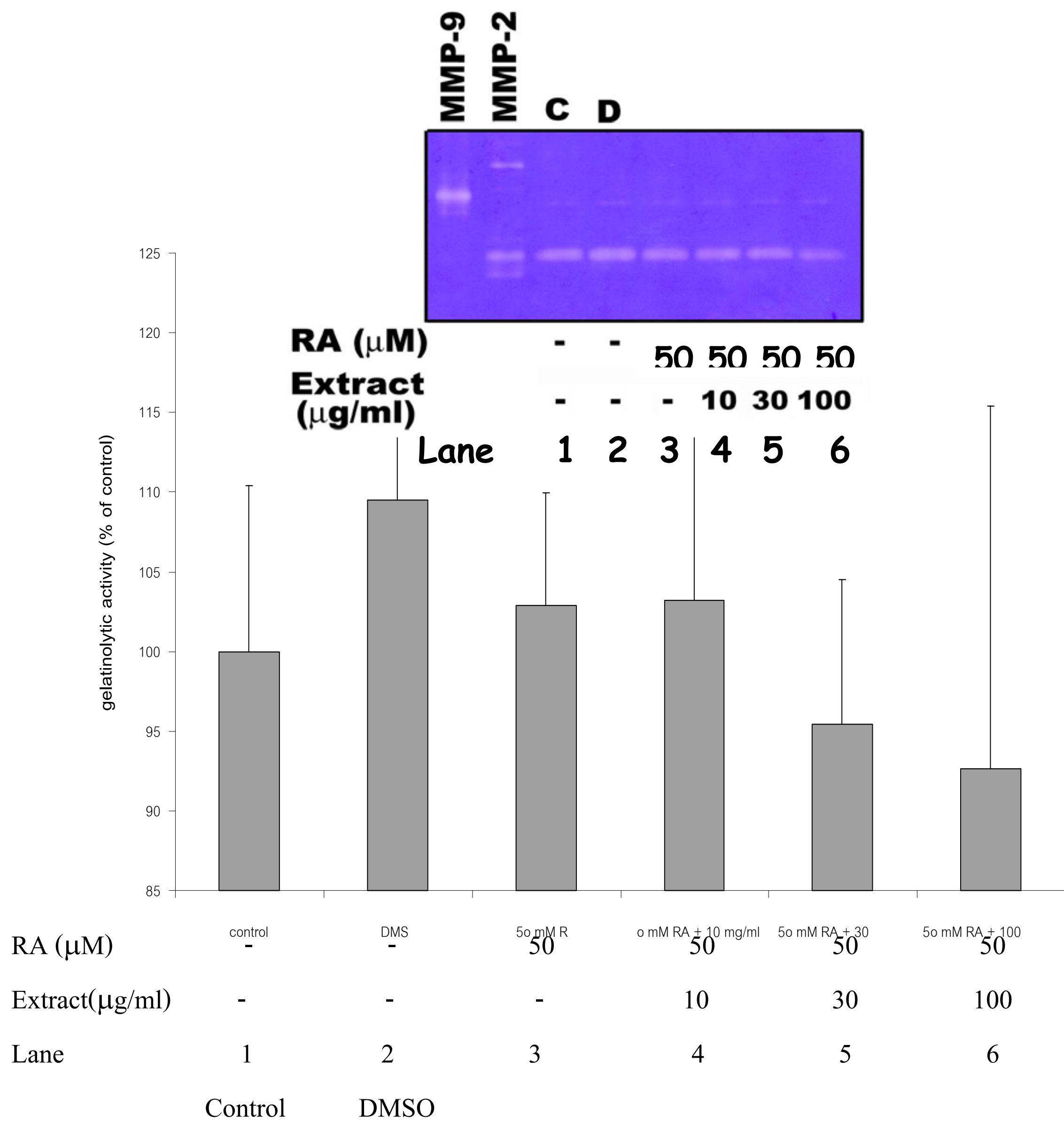

Figure 20. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral fibroblast medium. Cells were treated with 50 μM RA and the extract was added at doses 10, 30, 100 μg/ml, control (untreated), and solvent control (treated with DMSO), and 50 μM RA-treated control. Data shown are mean value ± standard deviation of triplicate assay per treatment.

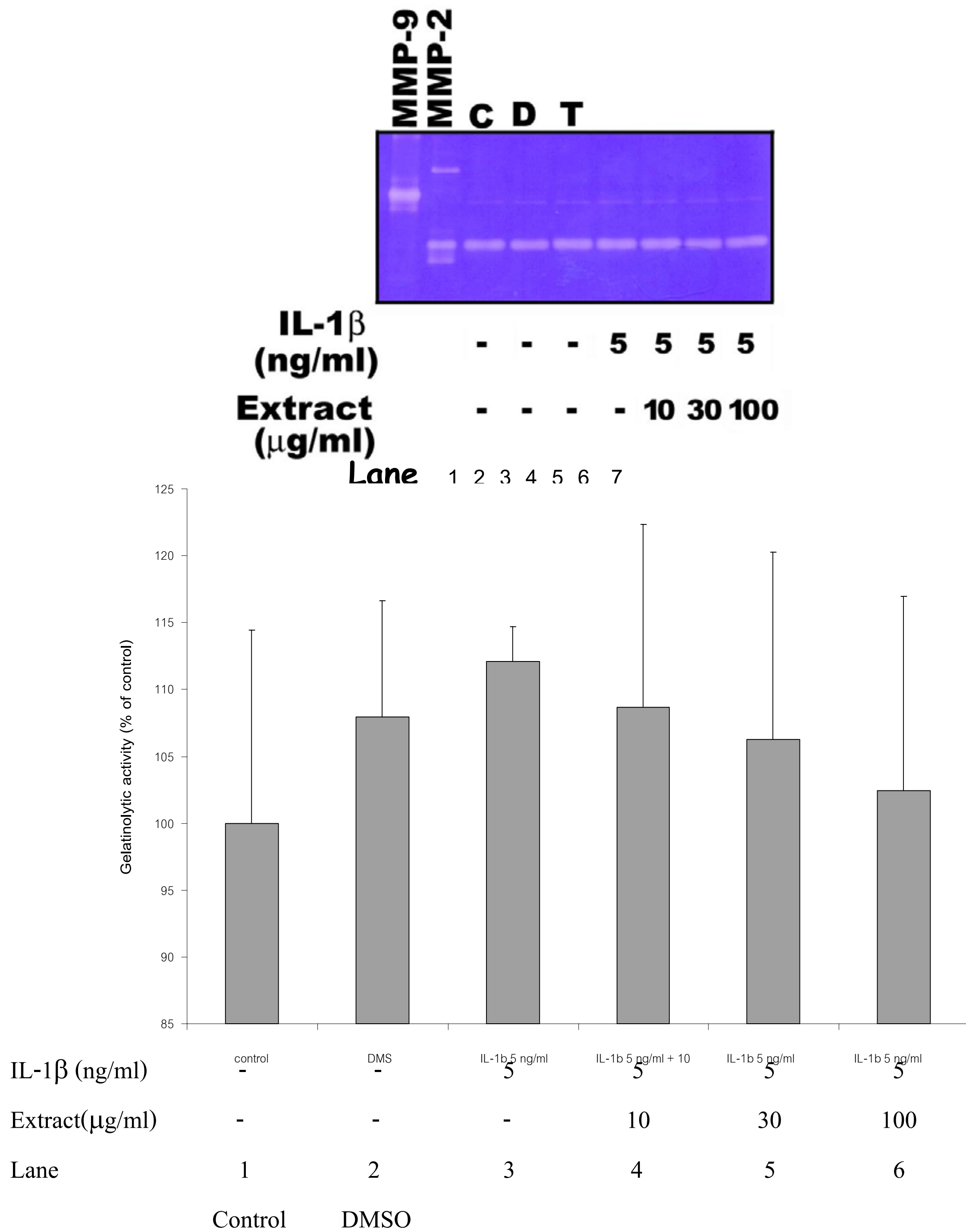

Figure 21. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral fibroblast medium. Cells were treated with 5 ng/ml IL-1β and the extract was added at doses 10, 30, 100 μg/ml, control (untreated), and solvent control (treated with DMSO), and 5 ng/ml IL-1β-treated control. Data shown are mean value ± standard deviation of triplicate assay per treatment.

3.2.6 Effects of *Zingiber cassumunar* Roxb. extract, Retinoic acid (RA), Interleukin-1β (IL-1β), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of MMP-2, -9 from oral epithelial medium.

In this study, activity of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in the culture media were investigated by gelatin zymography. Culture media were electrophoresed on native, non-reducing, gelatin containing gels, which were subsequently stained with Coomassie blue; resolved gelatinolytic proteins were detected as unstained band. Oral epithelia culture in serum-free medium secreted high levels of gelatinase, which are pro-MMP-2 and pro-MMP-9, when compared to standard enzymes and markers at 72 kDa and 92 kDa respectively.

The results showed that ethanol extracted Plai significantly down regulated the production of MMP-2 and MMP-9 in the oral epithelia in dose dependent manner, as shown in Figure 22.

Treatment with RA, IL-1β, TPA showed significantly up regulation of the production of MMP-9 in oral epithelium media. Co-treatment of RA, IL-1β, TPA and ethanol extract exhibited down regulated the production of MMP-9 as compare with treated-RA, IL-1β, TPA and down regulated the production of MMP-2 as compare with untreated control as shown in Figures 23-25.

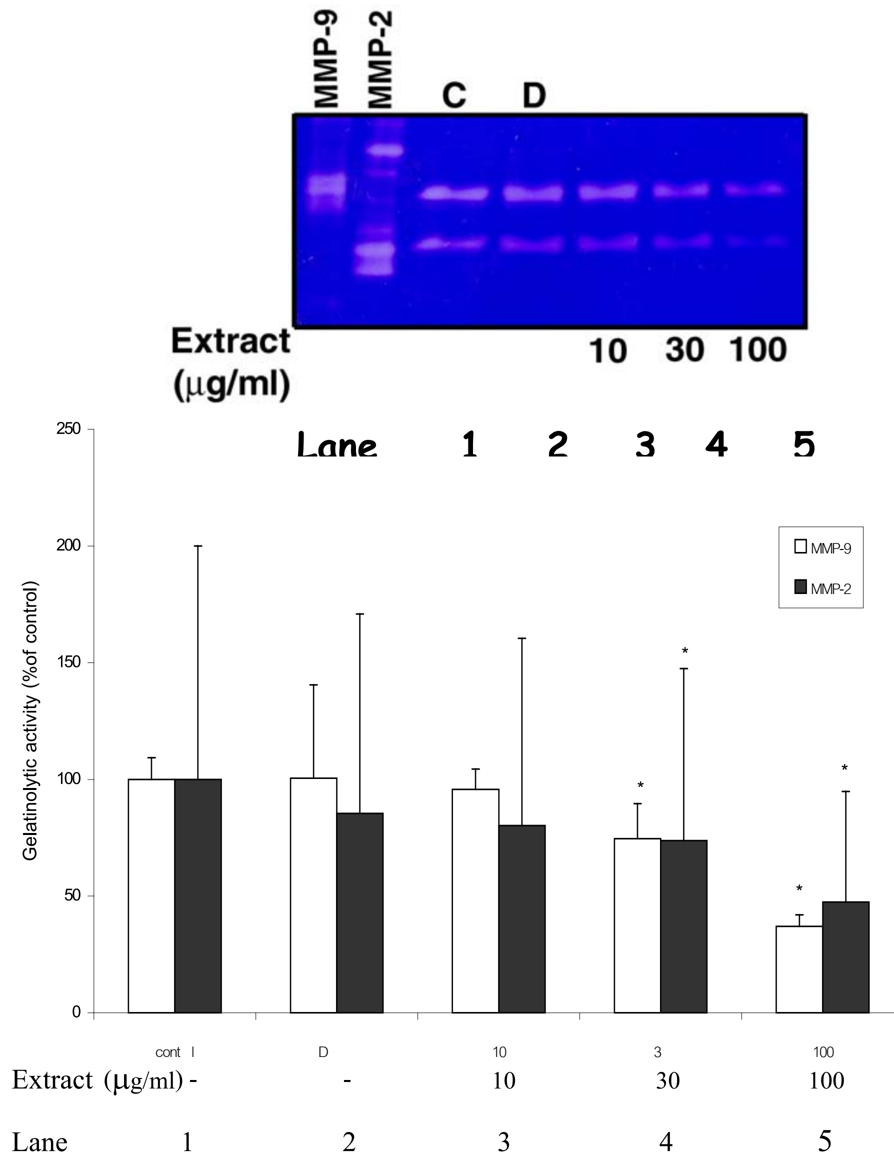

Figure 22. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral epithelial medium. Cells were treated with the extract at doses 10, 30, 100 µg/ml, control (untreated), and solvent control (treated with DMSO). Data shown are mean value $\pm$ standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

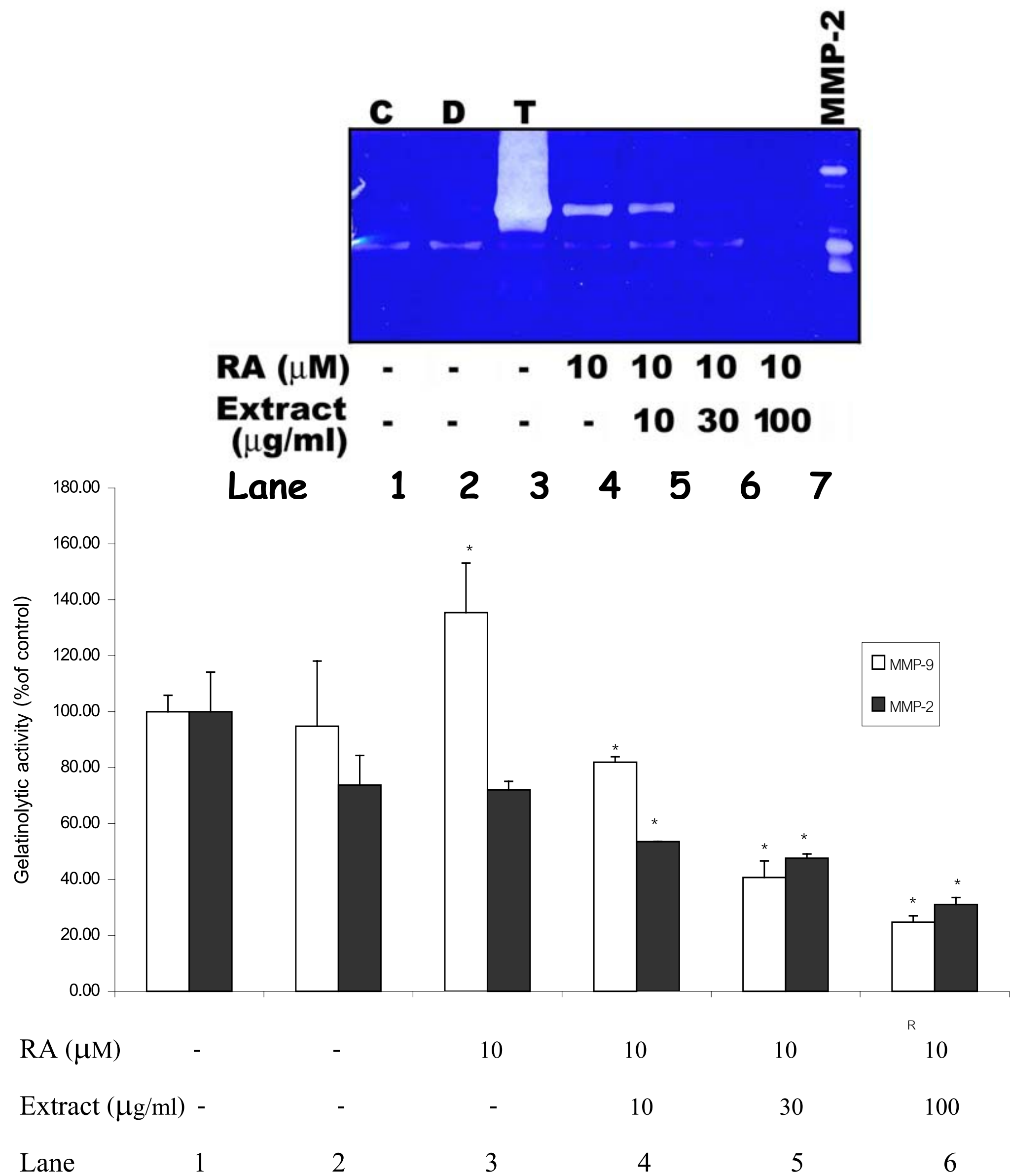

Figure 23. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral epithelial medium. Cells were treated with 10 μM RA and the extract was added at doses 10, 30, 100 μg/ml, 10 μM RA, control (untreated), and solvent control (treated with DMSO). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

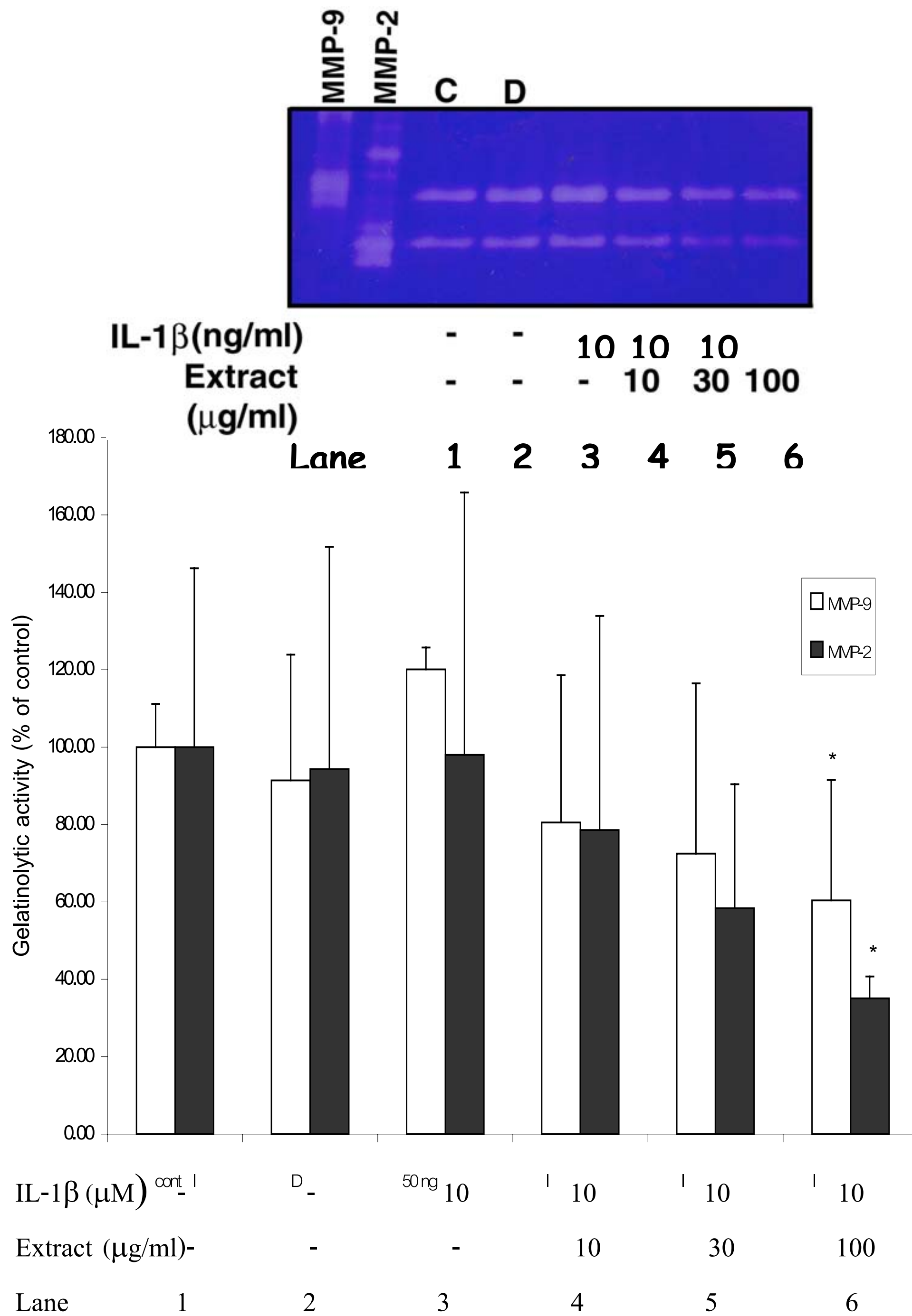

Figure 24. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral epithelial medium. Cells were treated with 10 ng/ml IL-1β and the extract was added at doses 10, 30, 100 μg/ml, 10 ng/ml IL-1β-treated control, control (untreated), and solvent control (treated with DMSO). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

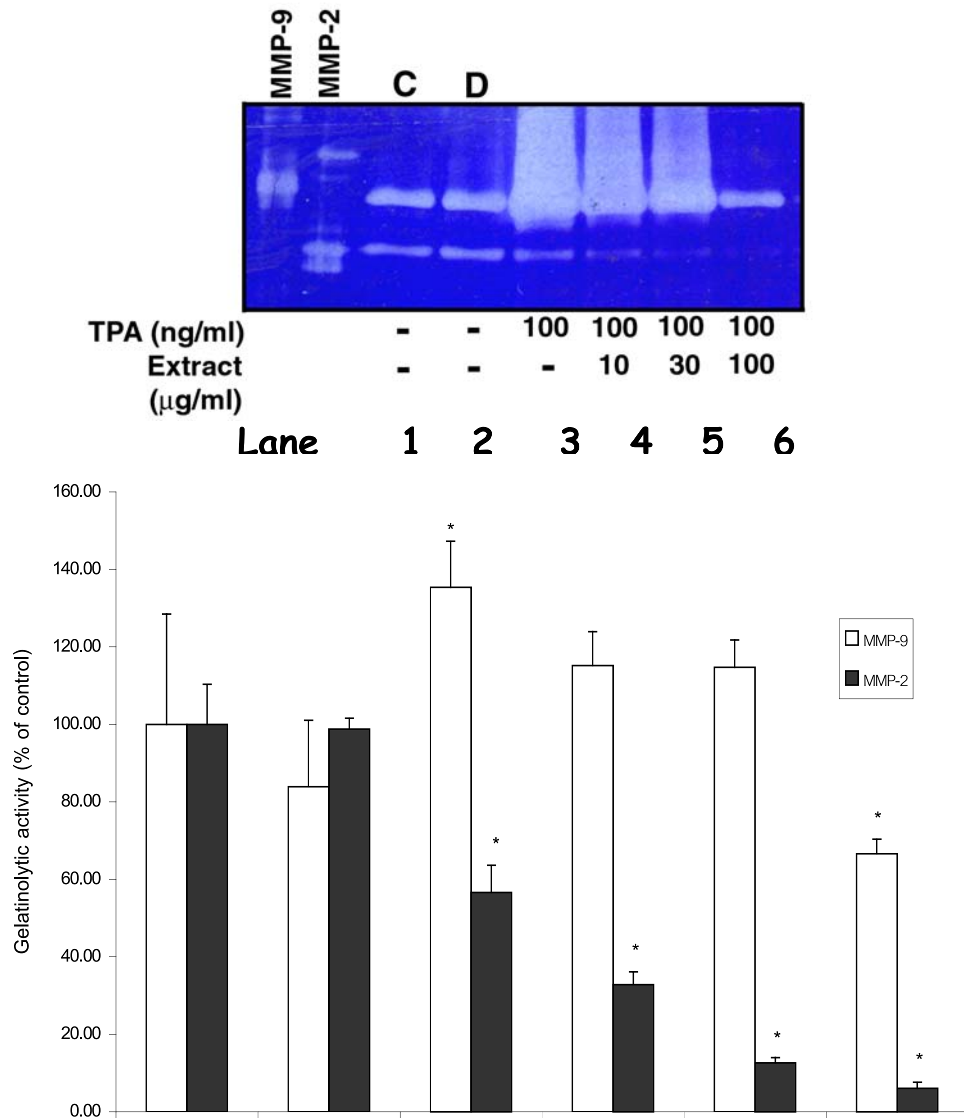

Figure 25. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral epithelial medium. Cells were treated with 100 ng/ml TPA and the extract was added at doses 10, 30, 100 μg/ml, 100 ng/ml TPA-treated control, control (untreated), and solvent control (treated with DMSO). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

3.3 Effects of *Zingiber cassumunar* Roxb. extract and Retinoic acid (RA) on cytotoxicity in oral fibroblast medium.

The cytotoxic of the extract to fibroblasts is base on the measurement of cytoplasmic enzyme activity releaesed by damaged cells. Oral fibroblasts were treated with RA at dose 0.1, 1.0, 10.0, 50.0 μM, or Plai ethanol extract at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, and/or combination between 10.0 μM RA and ethanol extracted Plai at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, 5.0 mM 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 media (RA, and Plai extract) were similar to the untreated control. The cytoplasmic enzyme activity released of the positive control was significantly increased by H_2O_2 (positive control) as showed in Figure 26.

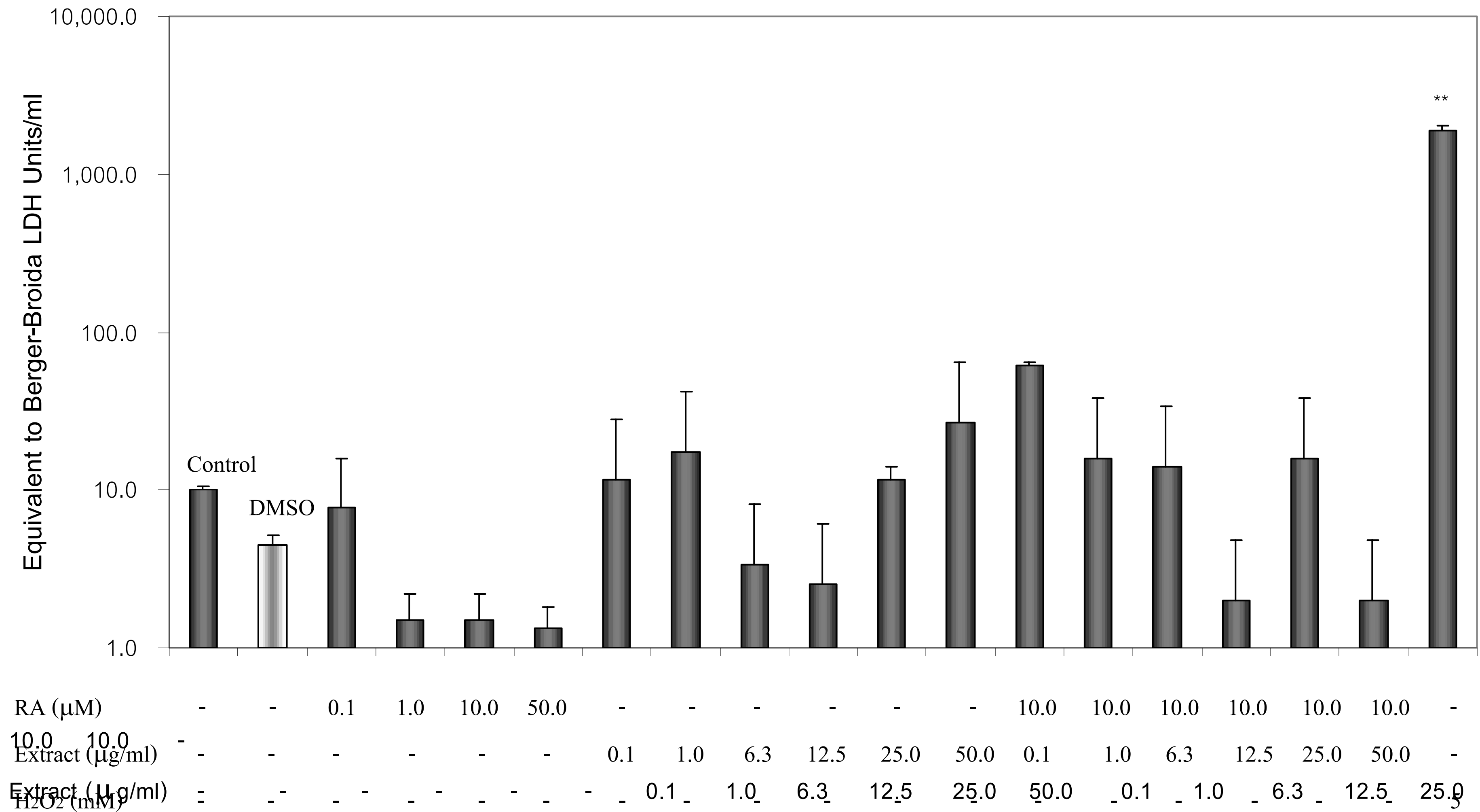

Figure 26. Effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on cytotoxicity in oral fibroblast medium. Cells were treated with 10 μM RA and the extract at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml, control (untreated), solvent control (treated with DMSO), and 0.1, 1, 10, 50 μM RA, 5.0 mM H_2O_2 (positive control). Data shown are mean value ± standard deviation of triplicate assay per treatment. ** Denoted values that were significantly different from untreated control, ($p<0.001$).

3.4 Effects of *Zingiber cassumunar* Roxb. extract and Retinoic acid (RA) on cytotoxicity in oral epithelial medium.

Oral epithelial cells were treated with RA at doses 0.1, 1.0, 10.0, 50.0 μM, ethanol extracted Plai at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, combination of 10.0 μM RA and ethanol extract at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, 5.0 mM 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 of treated culture media (RA, and Plai extract) were similar to the untreated control. The cytoplasmic enzyme activity released in positive control was significantly increased by H_2O_2 (positive control) as shown in Figure 27.

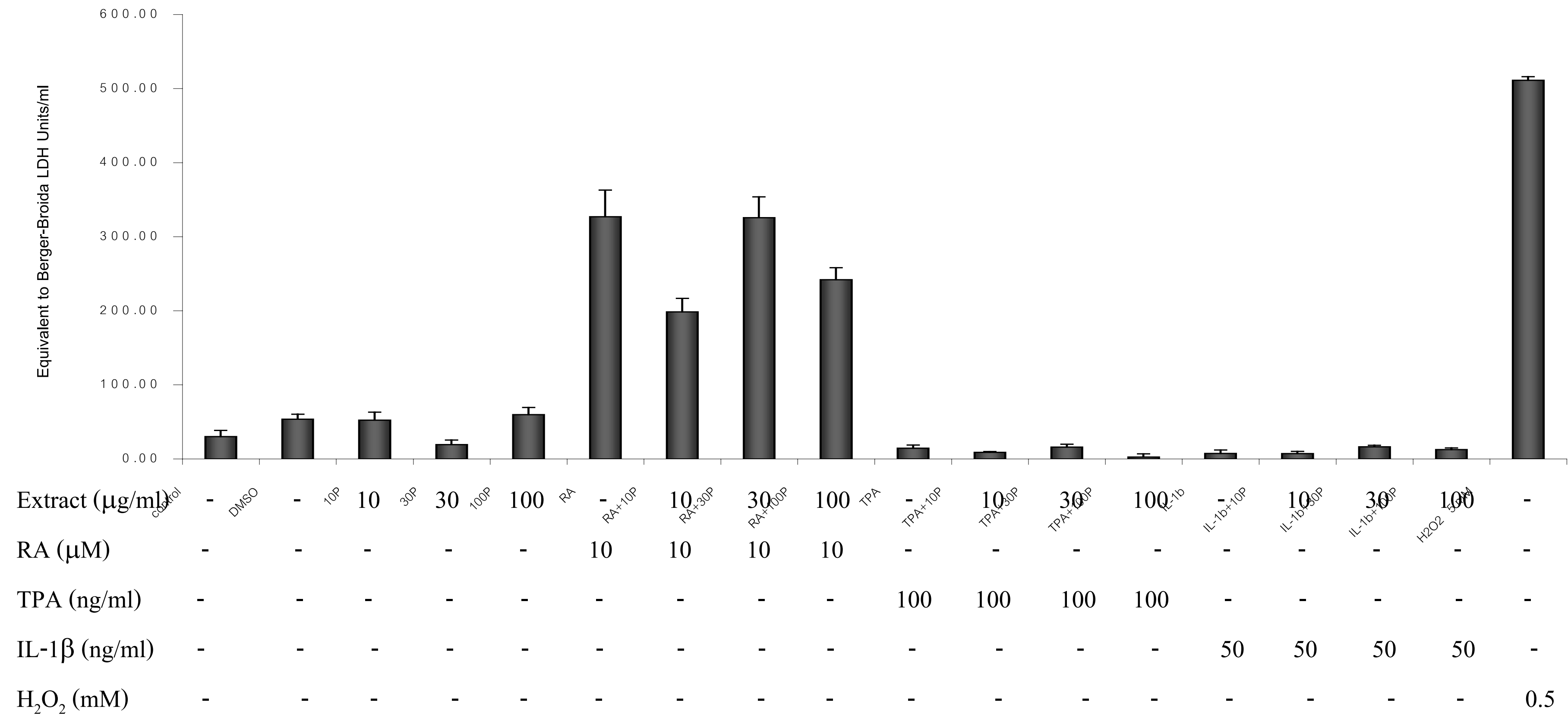

Figure 27. Effects of various concentrationsof the ethanol extract of *Zingiber cassumunar* Roxb. on cytotoxicity in oral epithelial medium. Cells were treated with 10 μM RA and the extract at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml, control (untreated), solvent control (treated with DMSO), and 0.1, 1, 10, 50 μM RA. Data shown are mean value ± standard deviation of triplicate assay per treatment. ** Denoted values that were significantly different from untreated control, ($p<0.001$).