CHAPTER 3

Results

3.1 Phytochemical screening

The phytochemical screening of ML extract revealed the compositions of alkaloids, phenolic and tannins, anthraquinone glycosides, flavonoids glycosides, cardiac glycosides, sterol glycosides or triterpene glycosides (Table 3.1).

Table 3.1 Phytochemical constituents of ML extract

Phytochemical test	Result	Confirmation
1. Alkaloid		
Dragendroff's reagent	+	+
Wagner's reagent	+	+
Mayer's reagent	+	+
2. Phenolic and tannin		
1% gelatin	+	
1% gelatin + salt	+	
1% $Fecl_3$	+	
3. Glycosides		
Antraquinone glycoside	+	
Flavonoid glycoside	+	
Saponin glycoside	-	
Cardiac glycoside	+	
Sterol glycoside	+	
Anthocyanin	-	

3.2 Determination of antioxidant activity of ML extract

3.2.1 Effect of ML extract on DPPH scavenging assay

The antioxidant activity of ML extracts was determined in the presence of the DPPH radical using spectrophotometry. Free radical scavenging activity is expressed as the percentage of DPPH decrease. The quality of the antioxidants in the extracts was determined as the % inhibition, denoting the concentration of the sample required to scavenge the DPPH free radicals. As shown in Figure 3.1, the antioxidant activity of the tested extracts increases with the quantity of raw material in the extract. The IC_{50} value is the concentration of extract required to scavenge the DPPH radical to 50% of the control. ML extract exhibited an IC_{50} value of 144 ± 22.5 µg/mL. As positive control, the IC_{50} value of gallic acid was 0.8 ± 0.16 µg/mL.

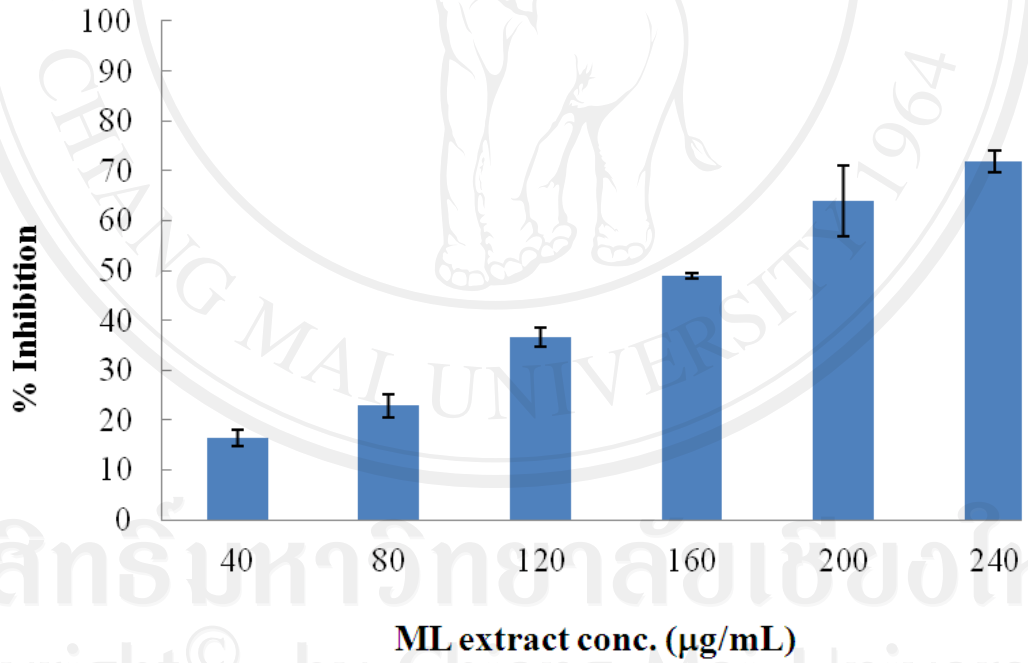


Figure 3.1 DPPH radicals scavenging activity of ML extract
Values are expressed as mean ± S.D (n=3) for three independent experiments

3.2.2 Total phenolic contents of ML extract

Total phenolics include all flavonoids, anthocyanins and nonflavonoid phenolic compounds. All the phenolic compounds present in the extracts were analyzed by Folin-Ciocalteau's method. The result for total phenolics was expressed as gallic acid equivalent (mg GAE/g). The standard graph of gallic acid is shown in Figure 3.2. The total content of phenolic compounds in ML extract is 197.2 ± 16.6 mg GAE/g dry extract.

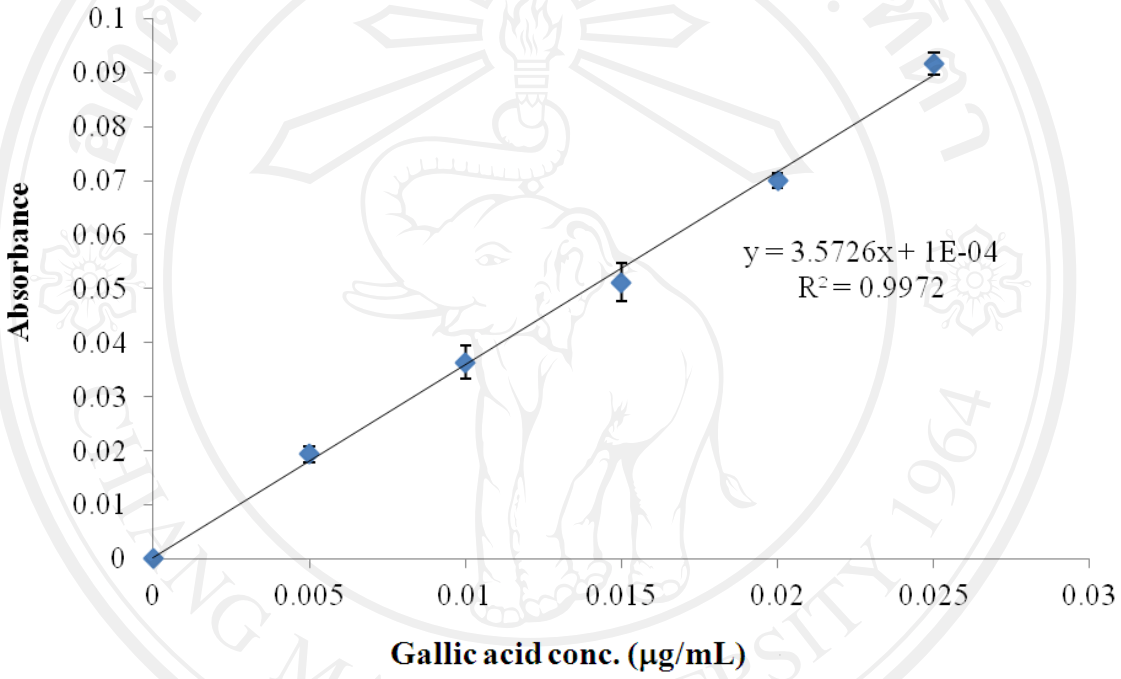


Figure 3.2 Gallic acid standard graph

3.3 *In vitro* anti-inflammatory models

3.3.1 Cell viability assay

ML extract at the concentrations of 3.125-50 µg/mL did not produce cytotoxic effect on RAW 264.7 cells. Viability was above 85% for 24 h incubation period. At 48 h incubated time, ML extract did not affect normal cell growth at concentrations of 3.125-12.5 µg/mL, with nearly 90% viability (Figure 3.3).

The effect of gallic acid (GA) on normal cell growth is shown in Figure 3.4. GA at the concentrations of 5-10 µg/mL did not exhibit cytotoxic effect on RAW 264.7 cells. This agent had viability above 90%.

From the results of cell viability assay, ML extract at the concentrations <50 µg/mL and GA at the concentrations <10 µg/mL were used for the next experiment.

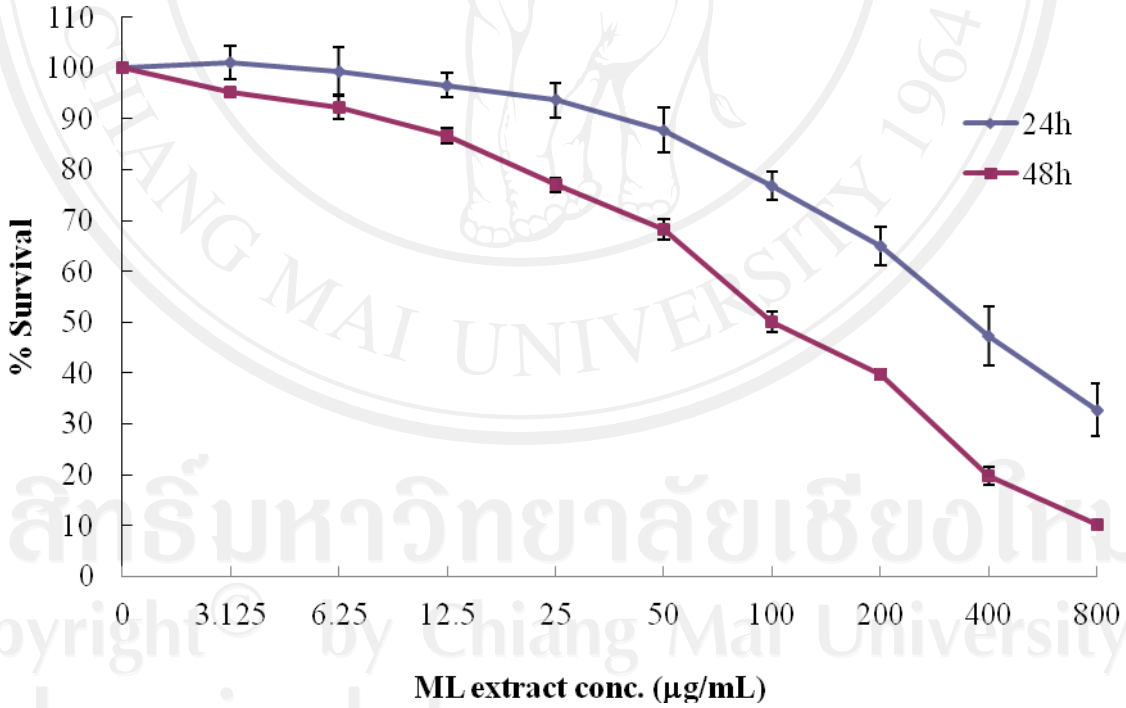


Figure 3.3 Effect of ML extract on viability of RAW 264.7 cells

Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

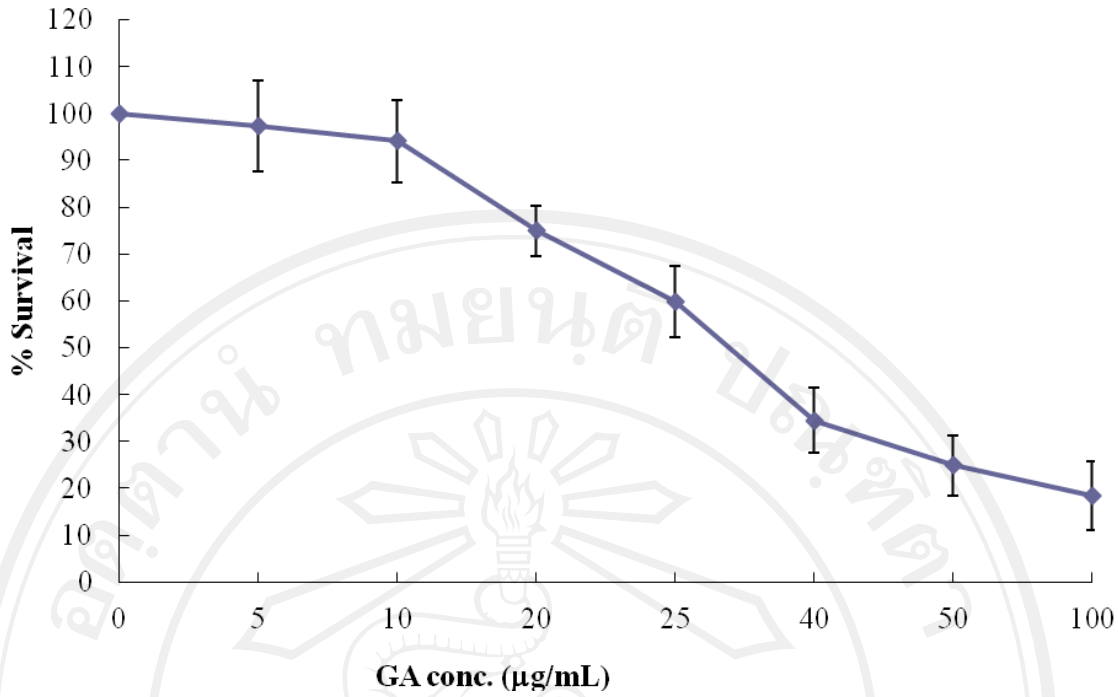


Figure 3.4 Effect of GA on viability of RAW 264.7 cells

Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

3.3.2 Nitrite assay

3.3.2.1 Inhibitory effects of GA on LPS-induced nitric oxide production in RAW 264.7 cells

The level of nitrite was determined in cultured media by the Griess reagent using the standard graph of sodium nitrite as shown in Figure 3.5. Cells pre-treatment with GA at the concentrations of 5, 10, 20, 25, 40 and 50 μg/mL significantly reduced the nitrite production in cultured supernatant (p<0.001). The nitrite levels decreased to 70-90% of the control (Figure 3.6). However, GA at the only concentrations of 5 and 10 μg/mL did not cause cells death. The other concentrations of GA significantly decreased the percent cells survival when compared with that of the control group (p<0.001). Thus, the effective concentrations of GA to reduce nitrite production were 5 and 10 μg/mL (Figure 3.7). GA at the concentration of 10 μg/mL was selected to use as a positive control in further experiments.

3.3.2.2 Inhibitory effects of ML extract on LPS-induced nitric oxide production in RAW 264.7 cells

To study the effect of ML extract on NO inhibition, RAW 264.7 cells were pre-treated with ML extract at the concentration of 0.5, 5 and 50 μg/mL before stimulating with LPS. The cultured media was collected for determination of nitrite levels at 24 and 48 h after LPS stimulation.

In pre-treatment experiment, ML extract at the concentration of 50 μg/mL, significantly decreased the nitrite levels when compared with the media control (p<0.001). ML extract reduced nitrite levels by 60% and 50% at 24 h and 48 h, respectively (Figure 3.8). ML extract showed inhibitory activity on nitrite production without affect the cell viability (Figures 3.9-3.10). The positive control, GA at the concentration of 10 μg/mL, showed inhibitory effects on nitrite production. GA reduced nitrite levels by 80 and 65% at 24 and 48 h, respectively (p<0.001). As shown in Figure 3.11, ML extract did not reduce the elevation of nitrite levels at any time in the post-treatment

experiment. However, GA significantly reduced nitrite concentration in post-treatment experiment. It reduced nitrite levels by 30%, 40%, and 20% at 2 h, 4 h and 6 h without effect on cell survival (Figure 3.12).

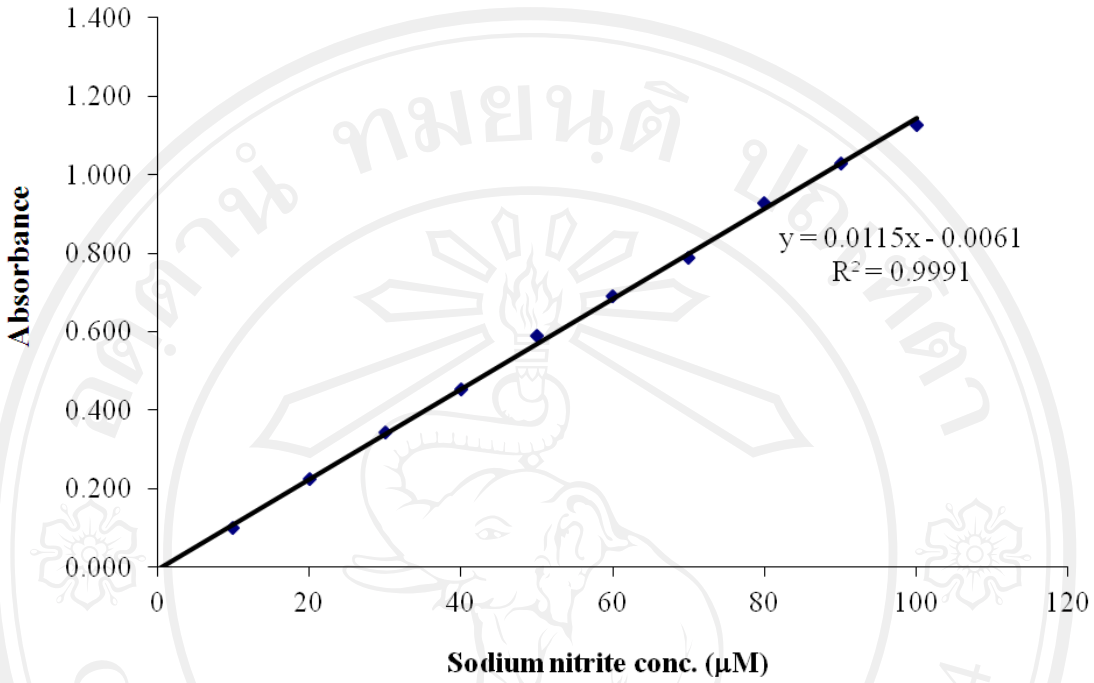


Figure 3.5 The standard curve of sodium nitrite

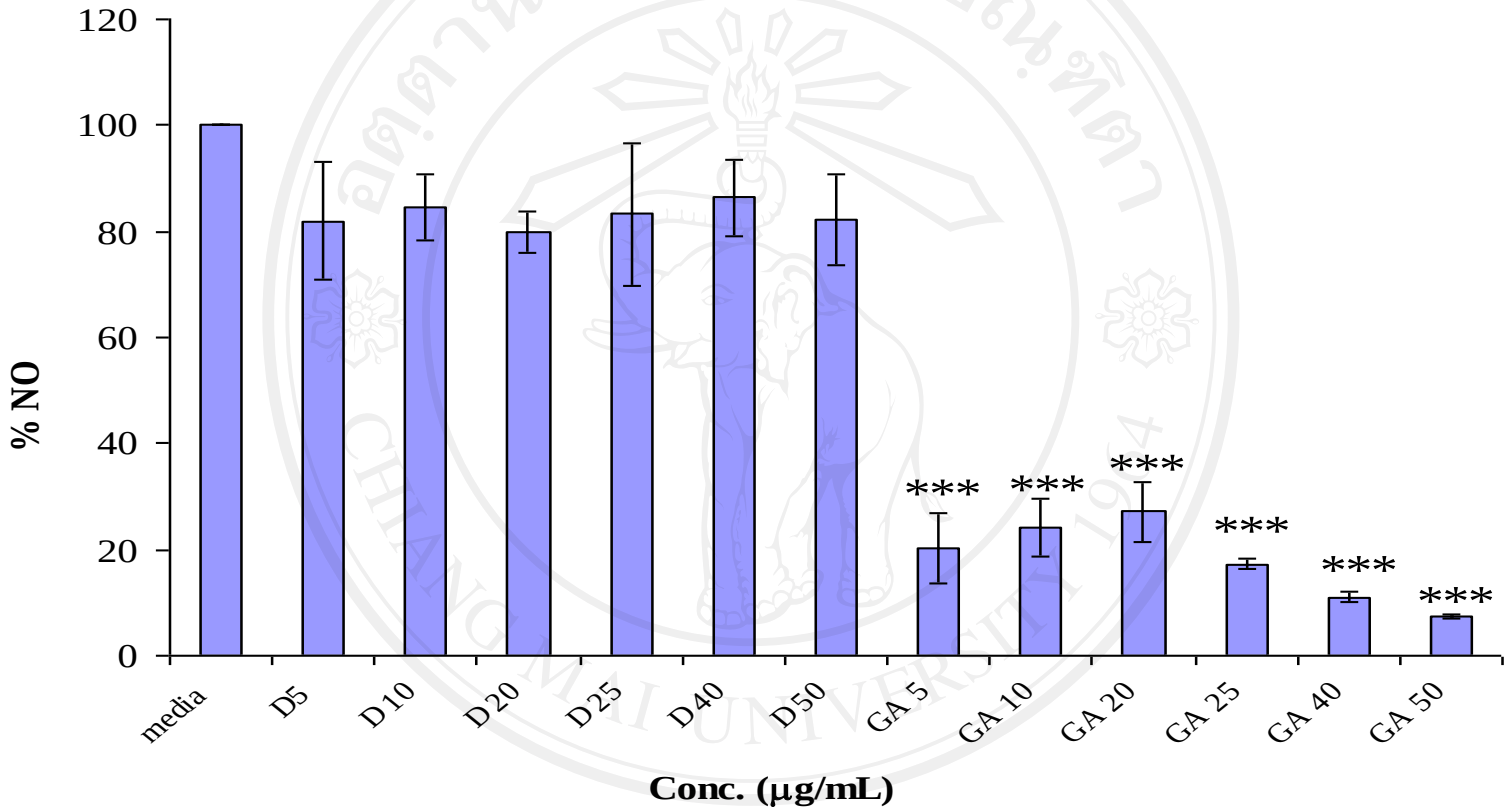


Figure 3.6 Inhibitory effects of GA on LPS-induced NO production in RAW 264.7 cells

Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

*** Significantly different from the control group, $p<0.001$

D: DMSO, GA: gallic acid

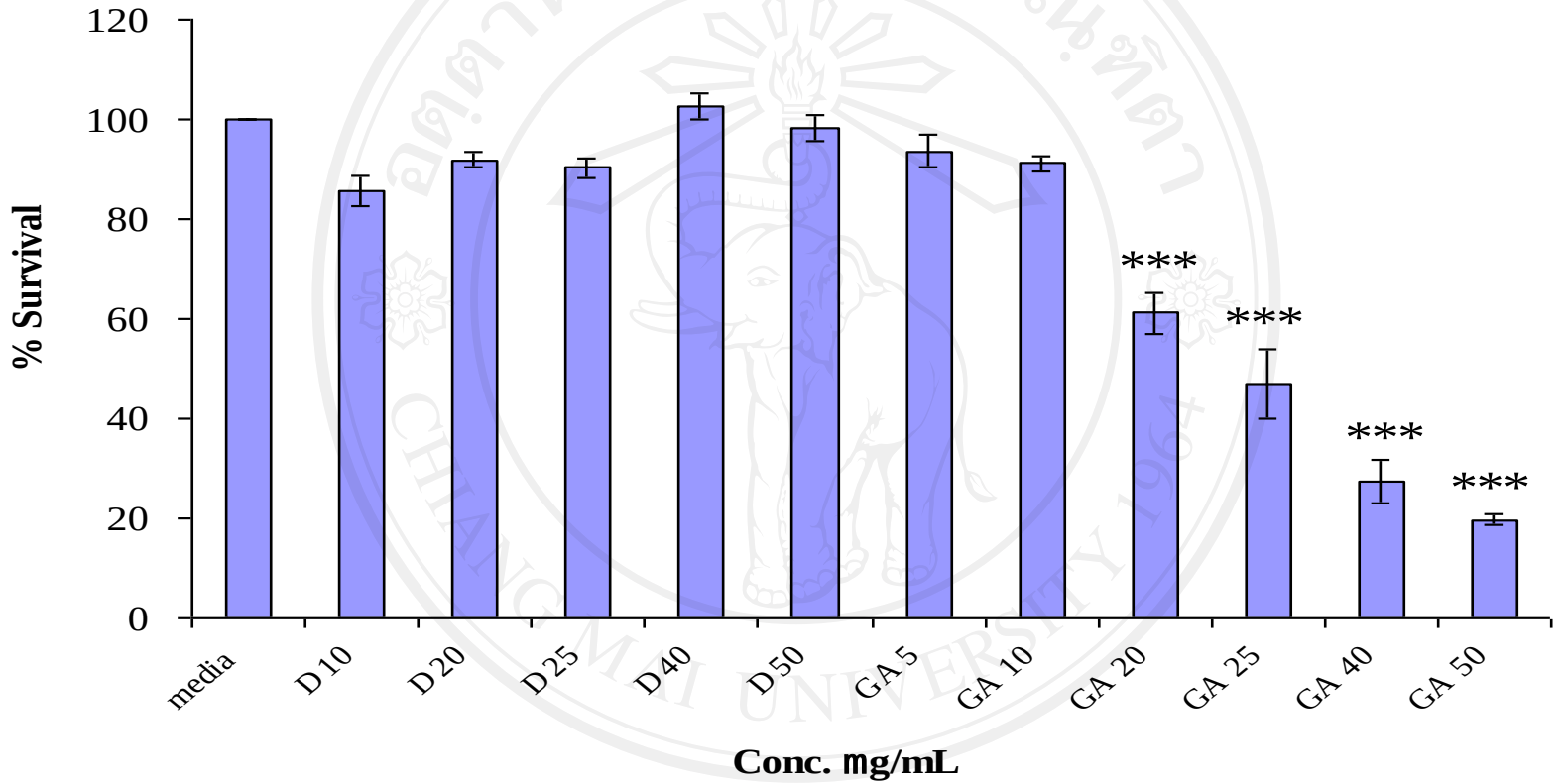


Figure 3.7 Effect of GA on the viability of RAW 264.7 cells after NO measurement

Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

*** Significantly different from the control group, $p<0.001$

D: DMSO, GA: gallic acid

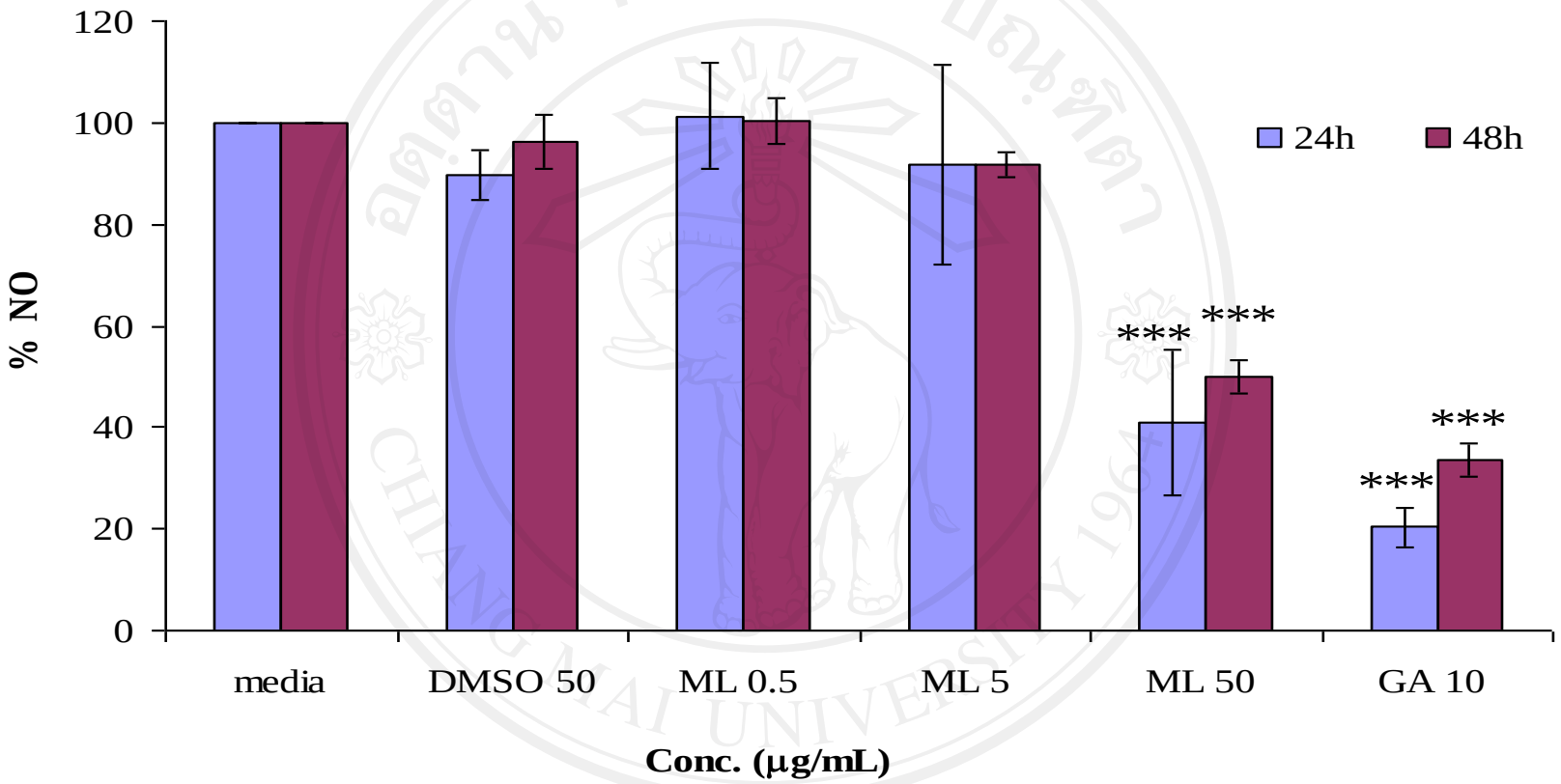


Figure 3.8 Effects of ML extract on LPS-induced nitric oxide production in RAW 264.7 cells (Pre-treatment experiment)

Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

*** Significantly different from the control group, $p<0.001$

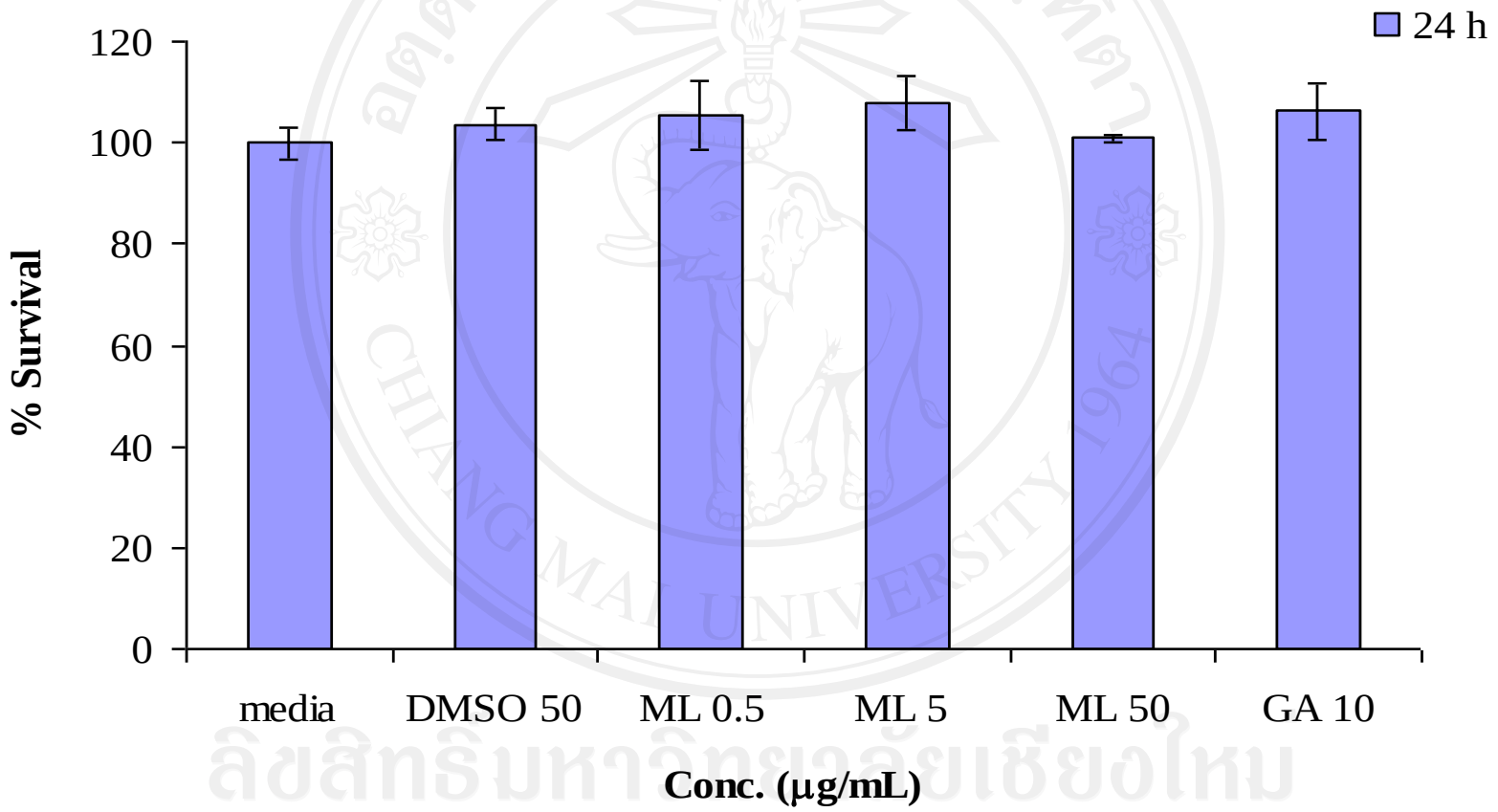


Figure 3.9 Viability of RAW 264.7 cells after NO assay (Pre-treatment experiment, 24 h)

Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

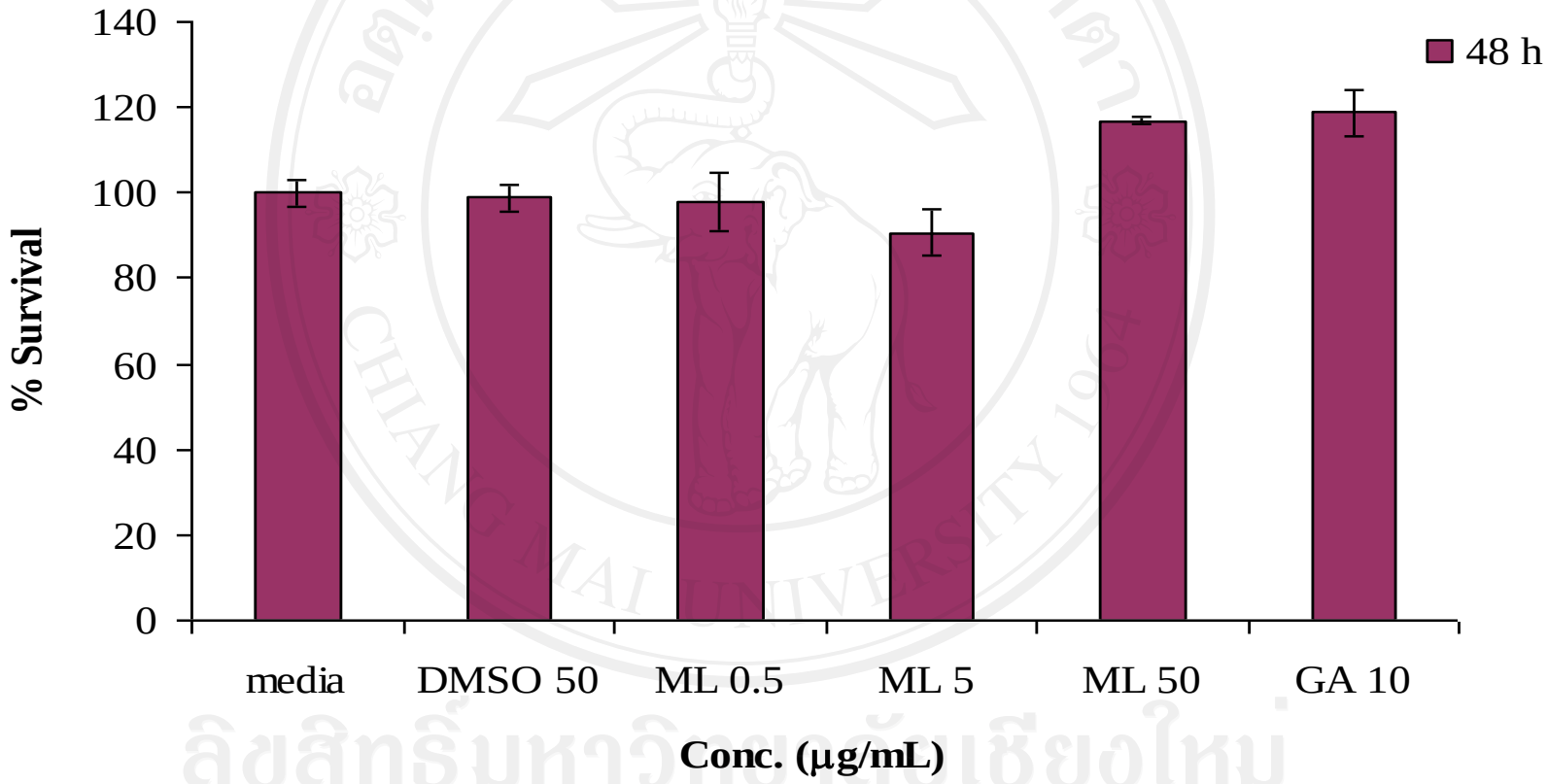


Figure 3.10 Viability of RAW 264.7 cells after NO assay (Pre-treatment experiment, 48 h)
Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

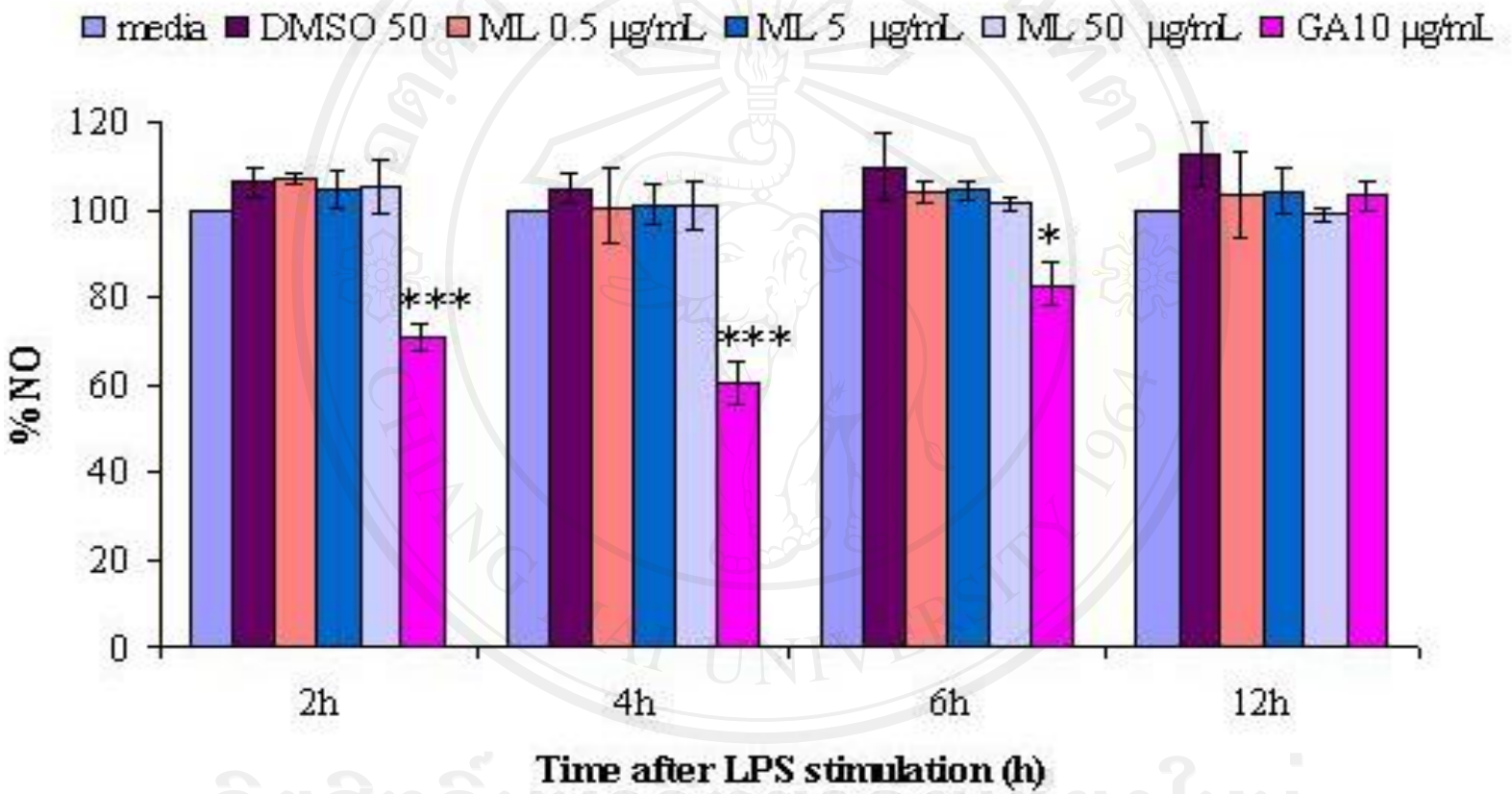


Figure 3.11 Effects of ML extract on LPS-induced nitrite production in RAW 264.7 cells (Post-treatment experiment)

Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

* Significantly different from the control group, $p<0.05$, *** Significantly different from the control group, $p<0.001$

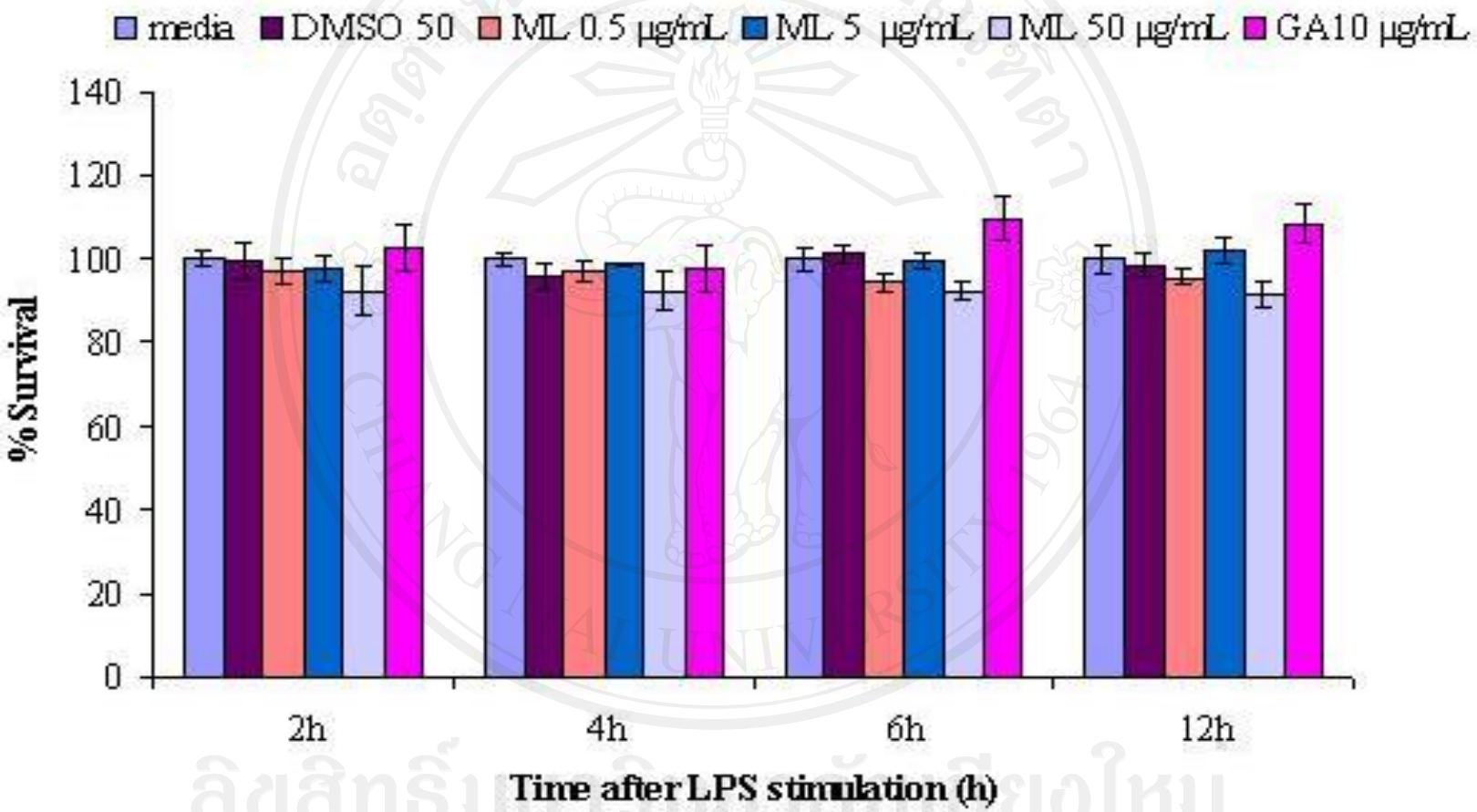


Figure 3.12 Viability of RAW 264.7 cells after NO assay measurement (Post-treatment experiment)
Values are expressed as mean ± S.E.M. (n=3) for three independent experiments

3.3.3 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

3.3.3.1 Effects of ML extract on the suppression of iNOS, COX-2, IL-1β, IL-6 and TNF-α expression in LPS-stimulated RAW 264.7 cells

Treatment of LPS (1 μg/mL) into the culture of RAW 264.7 cells for 6 h markedly increased the expression of iNOS, COX-2, IL-1β, IL-6 and TNF-α. Cells pretreated with 50 μg/mL of ML extract suppressed the expression of these genes. Similar to GA, ML extract showed a significant reduction of inflammatory enzyme and pro-inflammatory cytokines gene expression (p<0.05). The obtained results are shown in Figure 3.13.

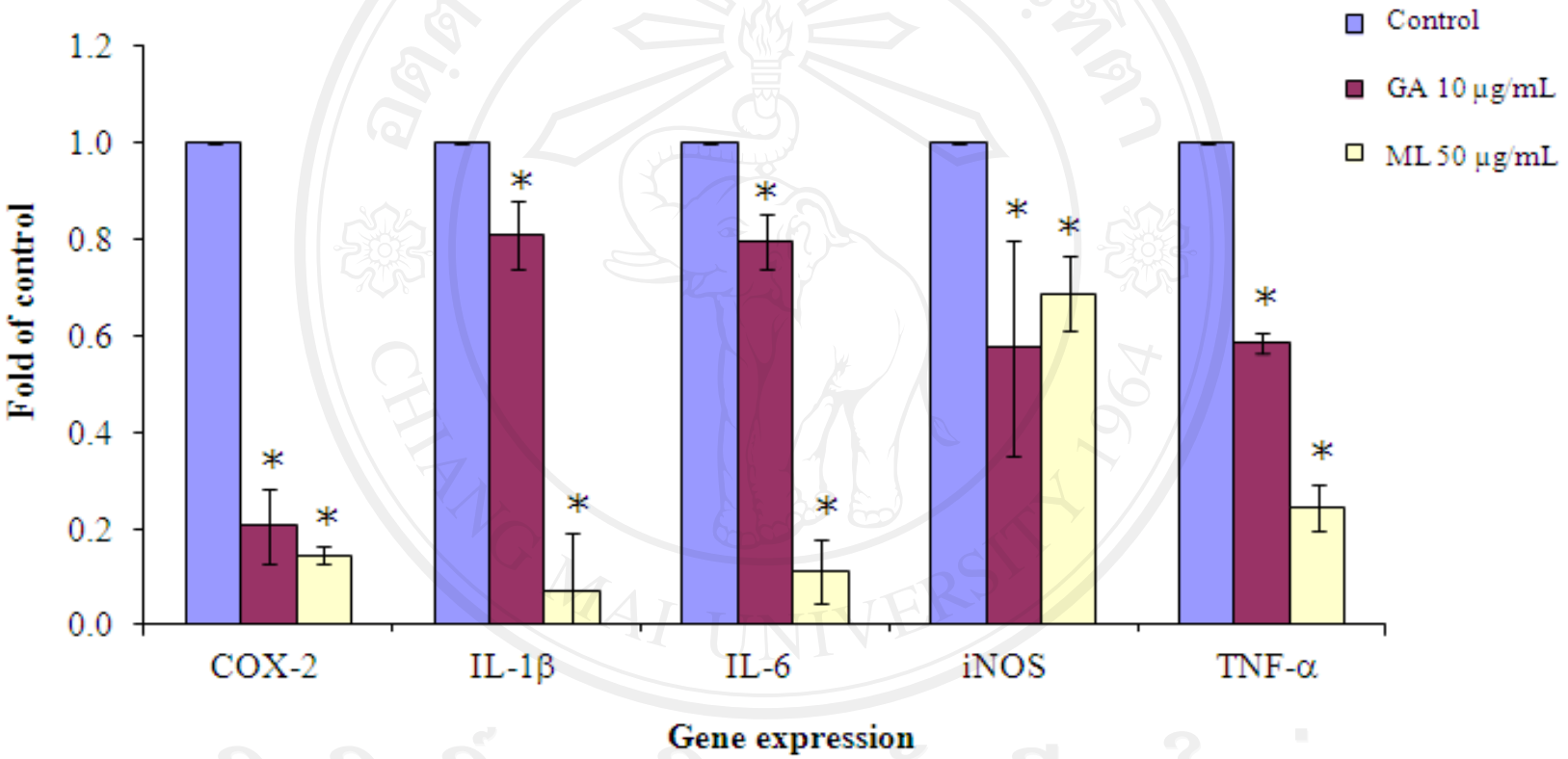


Figure 3.13 Effect of ML extract on inflammatory genes expression in RAW264.7 cells

The data are expressed as mean ± S.E.M. from three separated experiments

* Significantly different from the control group, $p<0.05$

3.4 *In vivo* experimental models

3.4.1 Anti-arthritic activity of ML extract

On the day 7th to the day 42nd after CFA injection, the injected right paws of all rats were swollen and appeared red. The right paw volume was significantly increased when compared with that of the left paws as shown in Figure 3.14.

The results of anti-arthritic activity of ML extract are illustrated in Figures 3.15-3.17. The control group showed the primary lesion of arthritis as the increasing of the injected right paw volume. The rat received ML extract (200 and 400 mg/kg) and indomethacin decreased the paw volume significantly when compared with that of the control group. The paw edema inhibition was observed on the day 7th, 14th, 21st and 28th, respectively (Figure 3.16). The inhibitory effect of ML extract at the doses of 200 and 400 mg/kg on the right paw edema was comparable to the reference drug, indomethacin.

The result of the secondary lesion of arthritis (non-injected, left paw) is shown in Figure 3.17. The paw volume of the rat in control group was 1.55, 1.60, 1.70, 1.75, and 1.75 mL on the day 0, 7th, 14th, 21st and 28th, respectively. Both indomethacin and ML extract (400 mg/kg) significantly inhibited left paw edema on day 14th, 21st and 28th. Anti-edema effect of ML extract (400 mg/kg) on the left paw was similar to that of the indomethacin-treated group.

The body weight of the rats during the study is shown in Figure 3.18 and Table 3.2. The body weight gain of the rats was not different among groups. ML extract showed anti-edema on rat paws without effect on weight gain.

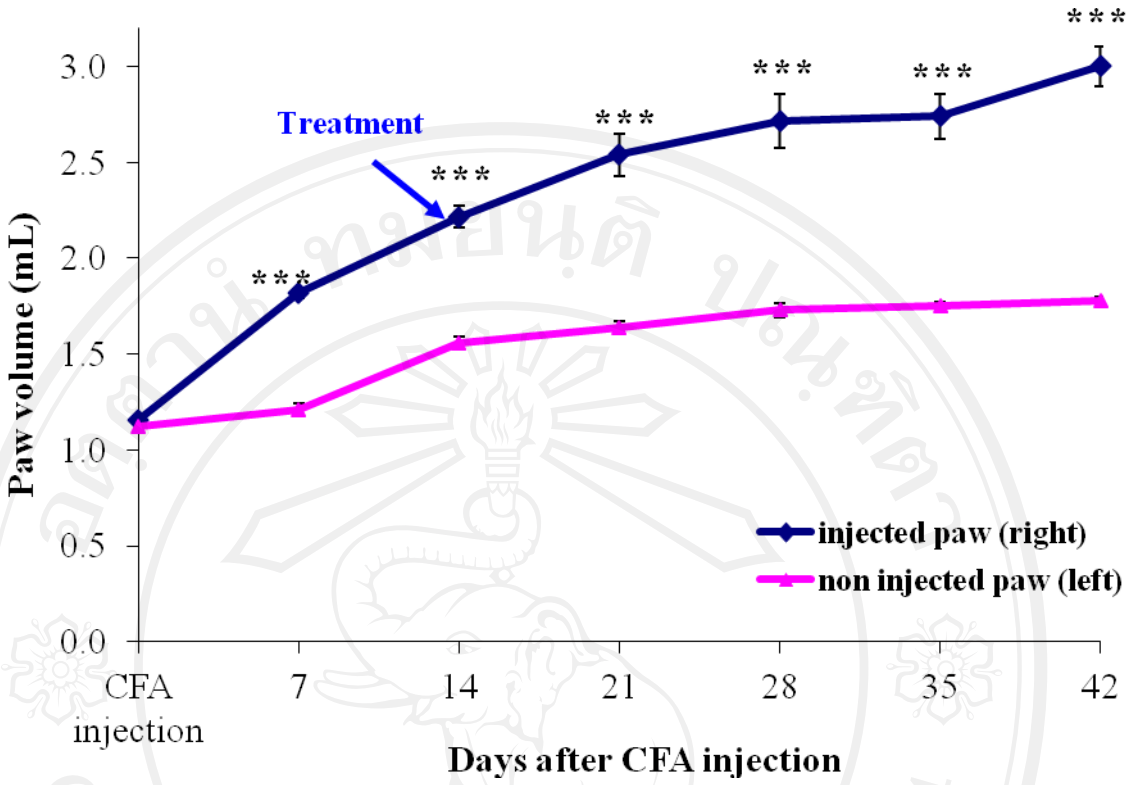
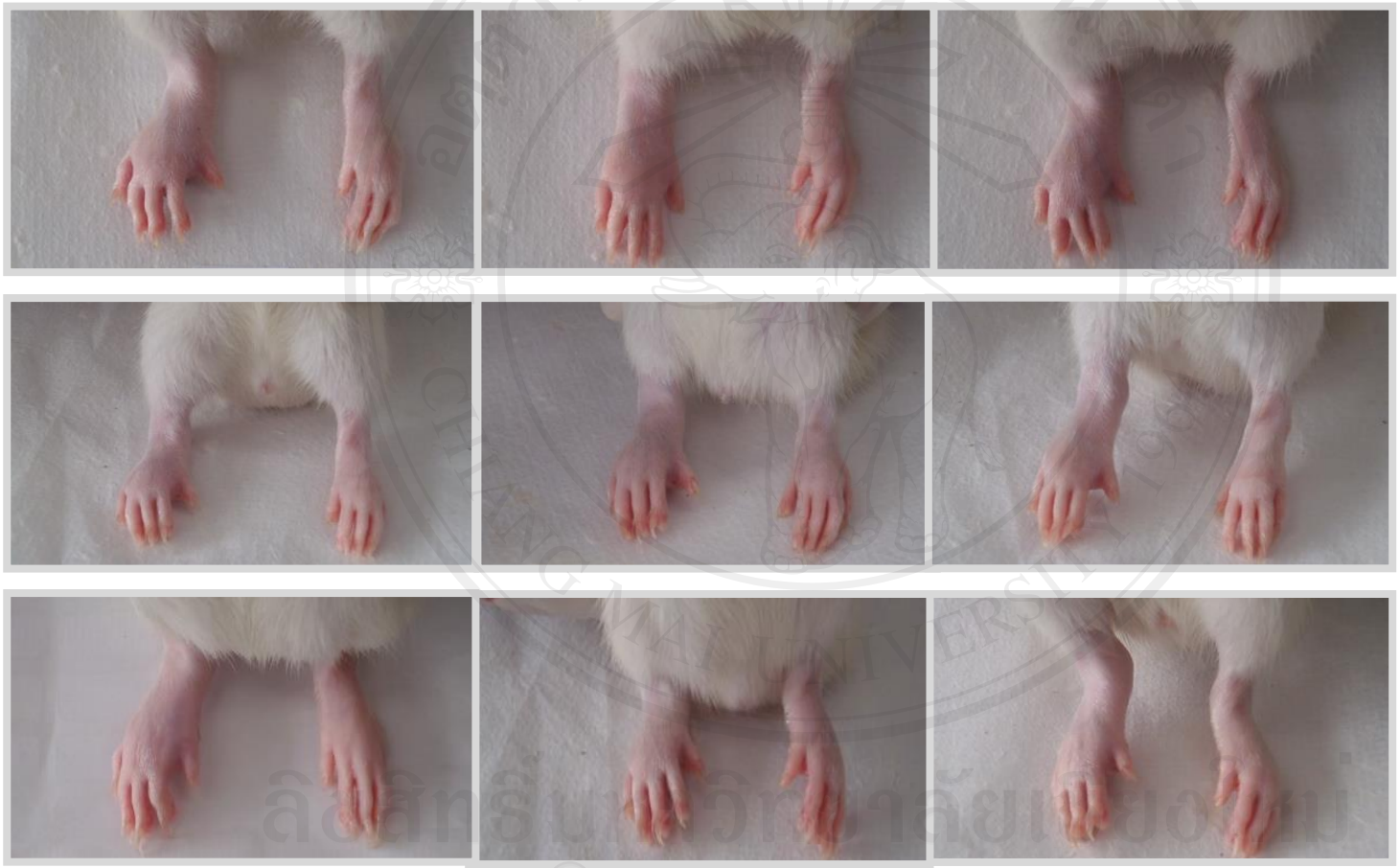


Figure 3.14 Time-course of paw volume of both injected and non-injected paws of arthritic rats

*** Significantly different from the non injected paw, $p<0.001$



Control group

Reference group (Indomethacin 1 mg/kg)

Test group (ML extract 400 mg/kg)

Figure 3.15 The photographs of joint swelling of the rats in each group on the day 28th

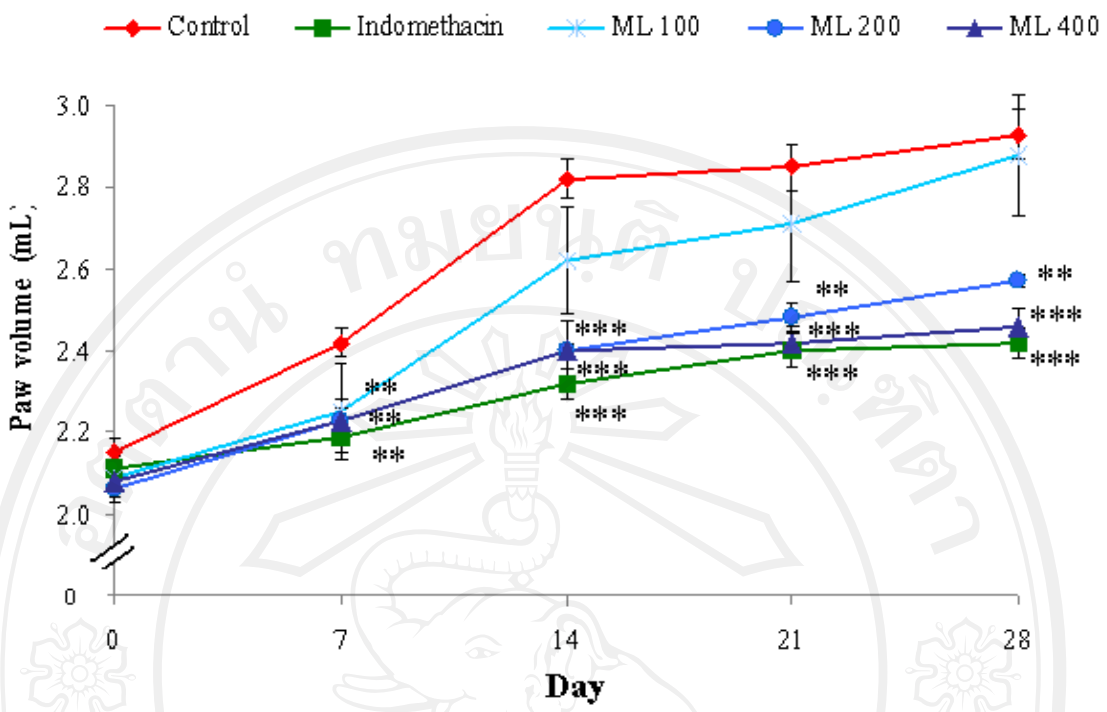


Figure 3.16 Effect of ML extract on the right paw volume in arthritic rats

Values are expressed as mean ± S.E.M. (n=6)

** Significantly different from the control group, $p<0.01$

*** Significantly different from the control group, $p<0.001$

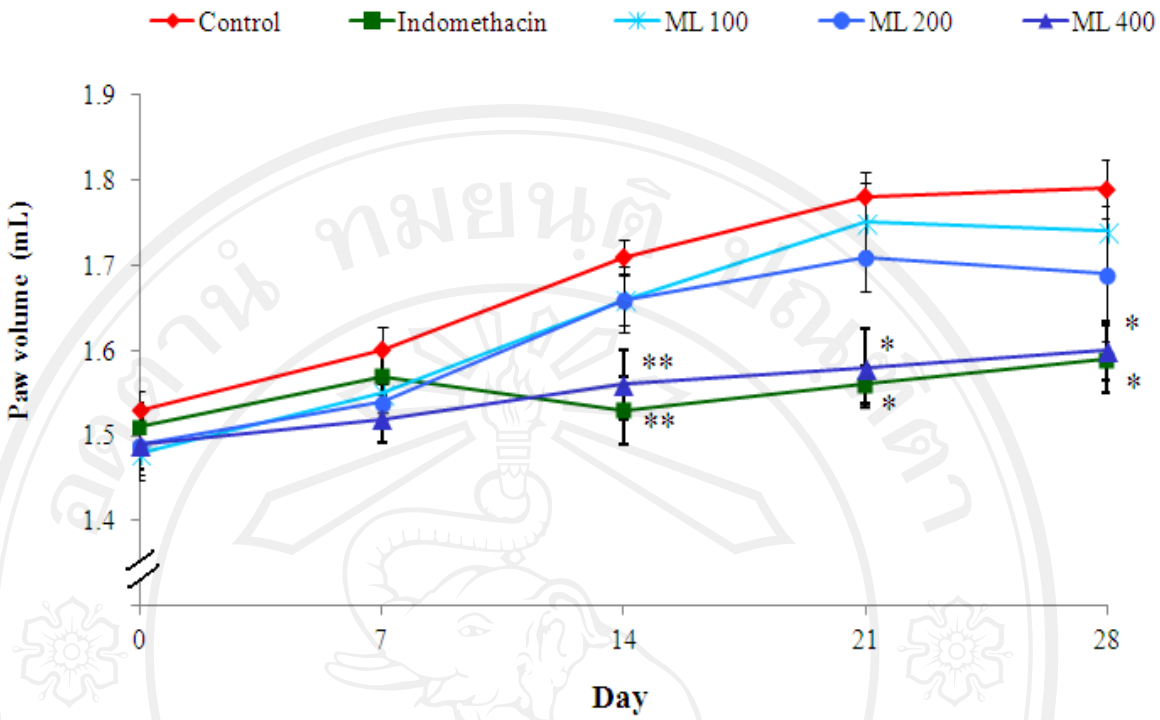


Figure 3.17 Effect of ML extract on the left paw volume in arthritic rats

Values are expressed as mean ± S.E.M. (n=6)

* Significantly different from the control group, $p<0.05$

** Significantly different from the control group, $p<0.01$

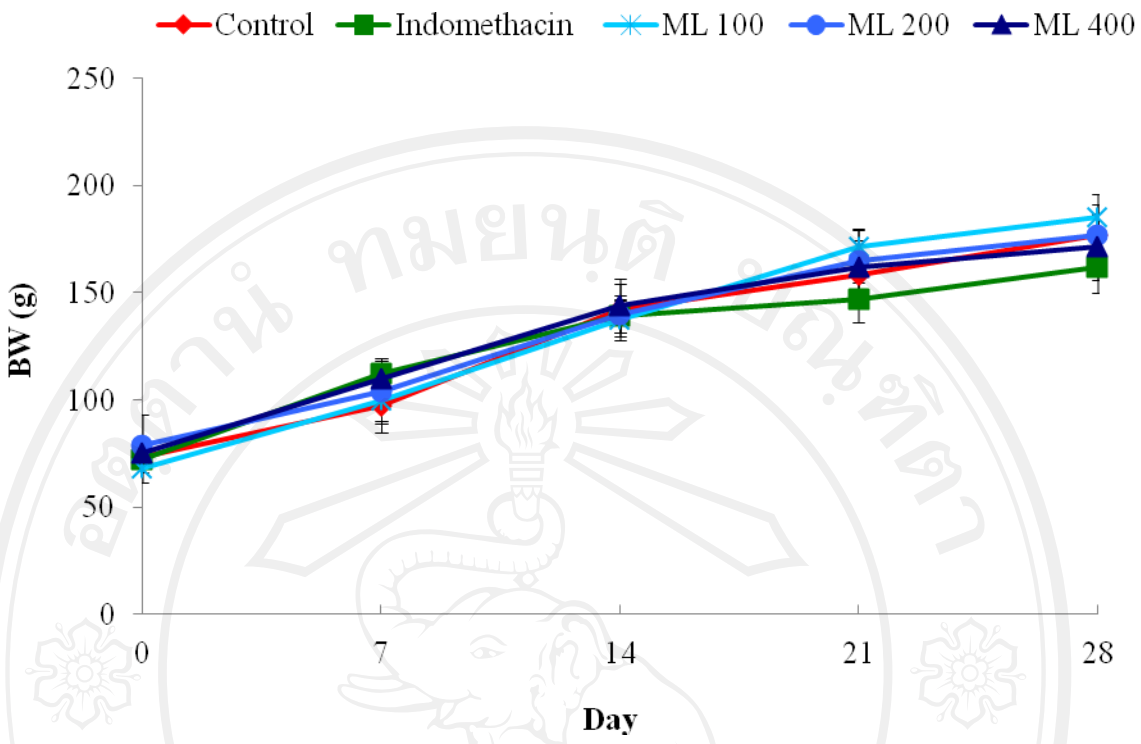


Figure 3.18 The body weight change of the rats in anti-arthritic activity study
Values are expressed as mean ± S.E.M. (n=6)

Table 3.2 Effect of ML extract on the total body weight gain of arthritic rats

Group	Dose (mg/kg)	Body weight (g)					
		Start	D0	D7	D14	D21	D28
Control	-	142.0 ± 5.15	215.4 ± 7.78	239.2± 5.68	284.0 ± 9.23	300.6 ± 11.20	318.8 ± 13.62
Indomethacin	1	142.5 ± 6.68	214.8 ± 9.77	255.0 ± 10.84	281.7 ± 13.91	289.2 ± 15.59	304.2 ± 17.58
ML	100	140.0 ± 6.38	208.8 ± 10.90	239.5 ± 11.32	277.5 ± 9.46	311.5 ± 10.72	325.0 ± 11.79
ML	200	130.5 ± 0.50	209.5 ± 4.11	234.8 ± 15.55	270.5 ± 8.58	295.5 ± 10.21	307.5 ± 13.77
ML	400	139.0 ± 7.02	214.2 ± 7.35	249.2 ± 6.60	283.0 ± 11.13	300.8 ± 10.30	310.5 ± 13.85

The rat body weight was measured at the started experiment day and on D0, D7, D14, D21 and D28

Values are expressed as mean ± S.E.M. (n=6)

3.4.1.1 Histological examination

The inflammatory cell infiltration and the proliferation of synovial membrane are the markers for tissue inflammation. The result from histological examination showed that indomethacin (1 mg/kg) and the ML extract (400 mg/kg) markedly reduced the infiltration of inflammatory cells into the ankle joint tissue. The extract and indomethacin also reduced the proliferation of synovial membrane when compared with that of the control group (Figure 3.19).

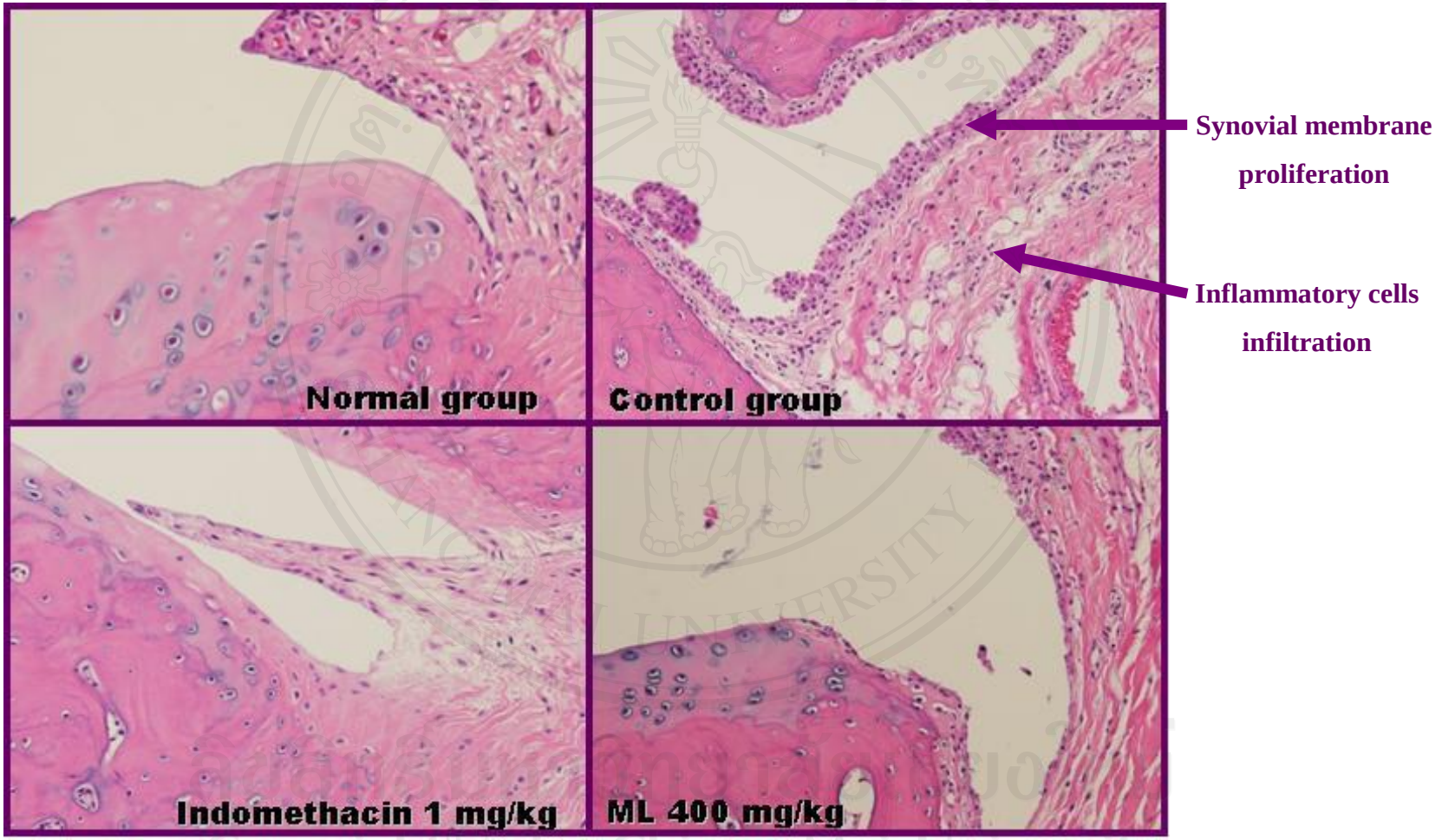


Figure 3.19 Histological images of ankle joints from the control and experimental groups of the rats

3.4.2 Effect of ML extract on EtOH/HCl-induced gastric ulcer in rats

Oral administration of EtOH/HCl caused severe gastric mucosal damage as illustrated in Figure 3.20. The reference drug, misoprostol showed highly effective in inhibition of gastric ulcer with 98% inhibition. In this model, ML extract dose-dependently and significantly inhibited gastric ulcer formation. The efficacy of the ML extract at the dose of 400 mg/kg on gastric ulcer formation was equivalent to that of misoprostol. The low dose of ML extract produced a 70% inhibition of gastric ulcer that was similar to that of the second reference drug, cimetidine (Table 3.3).

3.4.3 Effect of ML extract on indomethacin-induced gastric ulcer

As shown in Figure 3.21, the petechiae lesions in stomach were found after oral administration of indomethacin. Misoprostol and cimetidine showed gastric ulcer inhibitory effect in this model. The percent inhibition by misoprostol and cimetidine were 78 and 89, respectively. ML extract dose dependently and significantly inhibited gastric ulcer formation in this model. ML extract at the high dose of 400 mg/kg caused 81 percent inhibition of gastric ulcer (Table 3.4).

3.4.4 Effect of ML extract on restraint water immersion stress-induced gastric ulcer

In restraint water immersion stress-induced gastric ulcer model, hemorrhagic form of lesions was found in the glandular part of the stomach as shown in Figure 3.22. In this study, cimetidine at the dose of 100 mg/kg significantly reduced gastric ulcer formation by 89%. Similar to cimetidine, ML extract dose-dependently and significantly inhibited gastric ulcer formation when compared with vehicle control group. ML extract at the doses of 100, 200 and 400 mg/kg showed gastric ulcer inhibition by 58%, 69% and 76% respectively (Table 3.5).

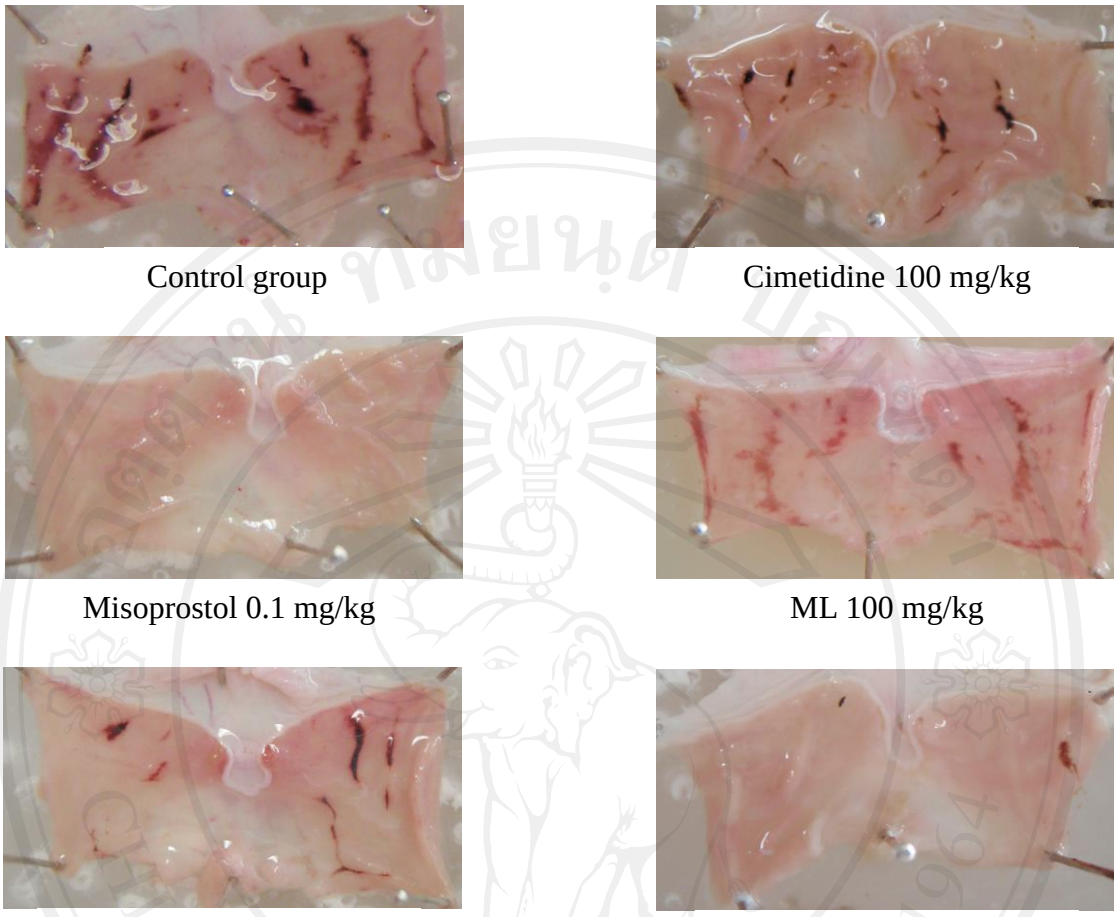


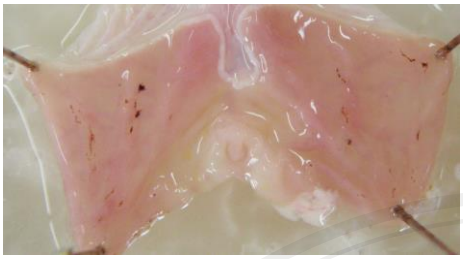
Figure 3.20 Macroscopic photographs of rat stomach with the acute gastric ulcer lesions induced by EtOH/HCl

Table 3.3 Effect of ML extract on EtOH/HCl-induced gastric ulcer in rats

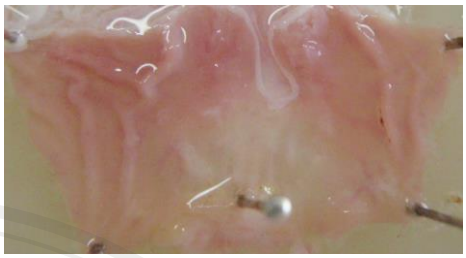
Group	Dose (mg/kg)	BW (g)	Ulcer (mm)	% Inhibition
Control	-	250.0 ± 6.95	84.2 ± 11.83	-
Cimetidine	100	251.0 ± 7.24	25.4 ± 3.56***	70
Misoprostol	0.1	250.8 ± 2.39	1.4 ± 0.86***	98
ML	100	252.5 ± 6.92	31.4 ± 3.88***	63
ML	200	254.2 ± 13.87	12.4 ± 7.28***	85
ML	400	252.0 ± 3.70	2.7 ± 2.22***	97

Values are expressed as mean ± S.E.M. (n=6)

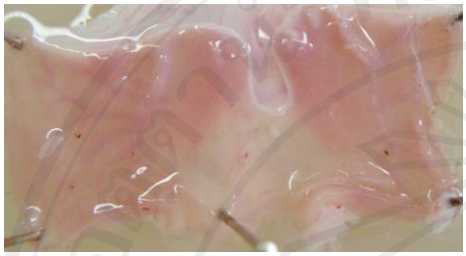
*** Significantly different from the control group, $p<0.001$



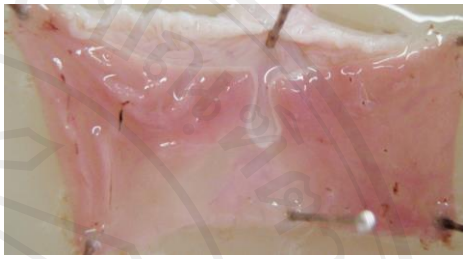
Control group



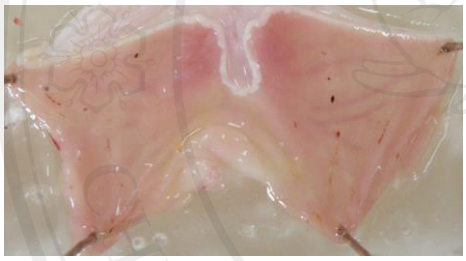
Cimetidine 100 mg/kg



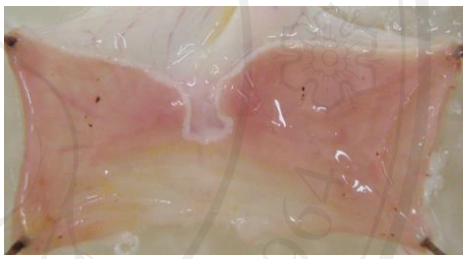
Misoprostol 0.1 mg/kg



ML 100 mg/kg



ML 200 mg/kg



ML 400 mg/kg

Figure 3.21 Macroscopic photographs of rat stomach with the acute gastric ulcer lesions induced by indomethacin

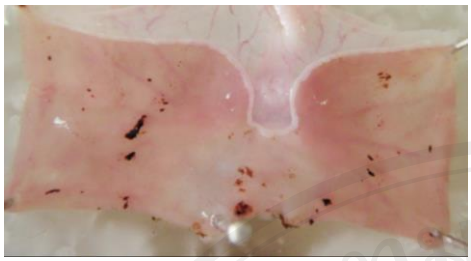
Table 3.4 Effect of ML extract on indomethacin-induced gastric ulcer

Group	Dose (mg/kg)	BW (g)	Ulcer (mm)	% Inhibition
Control	-	253.7 ± 6.54	7.02 ± 2.03	-
Cimetidine	100	251.5 ± 9.96	0.80 ± 0.48**	89
Misoprostol	0.1	253.3 ± 10.06	1.58 ± 0.74**	78
ML	100	253.0 ± 2.31	3.30 ± 1.53*	53
ML	200	250.8 ± 5.19	2.35 ± 0.94**	67
ML	400	251.5 ± 6.45	1.32 ± 0.57**	81

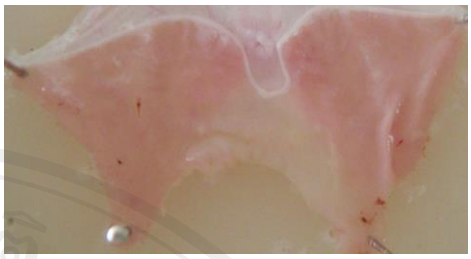
Values are expressed as mean ± S.E.M. (n=6)

* Significantly different from the control group, $p<0.05$

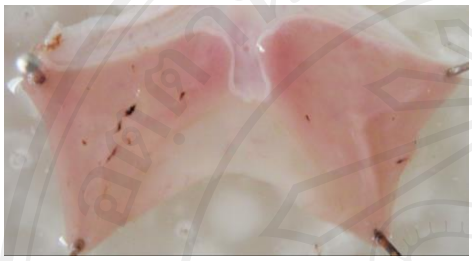
** Significantly different from the control group, $p<0.01$



Control group

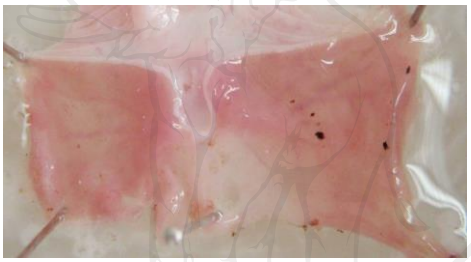


Cimetidine 100 mg/kg



ML 100 mg/kg

ML 200 mg/kg



ML 400 mg/kg

Figure 3.22 Macroscopic photographs of rat stomach with acute gastric ulcer lesions induced by stress

Table 3.5 Effect of ML extract on restraint water immersion stress-induced gastric ulcer

Group	Dose (mg/kg)	BW (g)	Ulcer (mm)	% Inhibition
Control	-	275.7 ± 3.94	11.15 ± 0.72	-
Cimetidine	100	273.7± 3.02	1.27 ± 0.35***	89
ML	100	269.8 ± 5.48	4.65 ± 1.05***	58
ML	200	273.8 ± 4.94	3.43 ± 1.04***	69
ML	400	273.7 ± 3.55	2.70 ± 0.50***	76

Values are expressed as mean ± S.E.M. (n=6).

*** Significantly different from the control group, $p<0.001$

3.4.5 Effect of ML extract on gastric visible mucus secretion

The standard curve of alcian blue is shown in Figure 3.23. The results of gastric wall mucus secretion are shown in Table 3.6. Gastric wall mucus in normal rats was 25.08 μg alcian blue/g wet stomach. ML extract alone did not change gastric wall mucus level when compared with the normal rat. Oral administration of HCl/EtOH significantly decreased gastric wall mucus in the control rats when compared with the normal rats. ML extract (400 mg/kg) and misoprostol significantly increased gastric wall mucus when compared with the control group. In this model, cimetidine was not effective in increasing gastric wall mucus level.

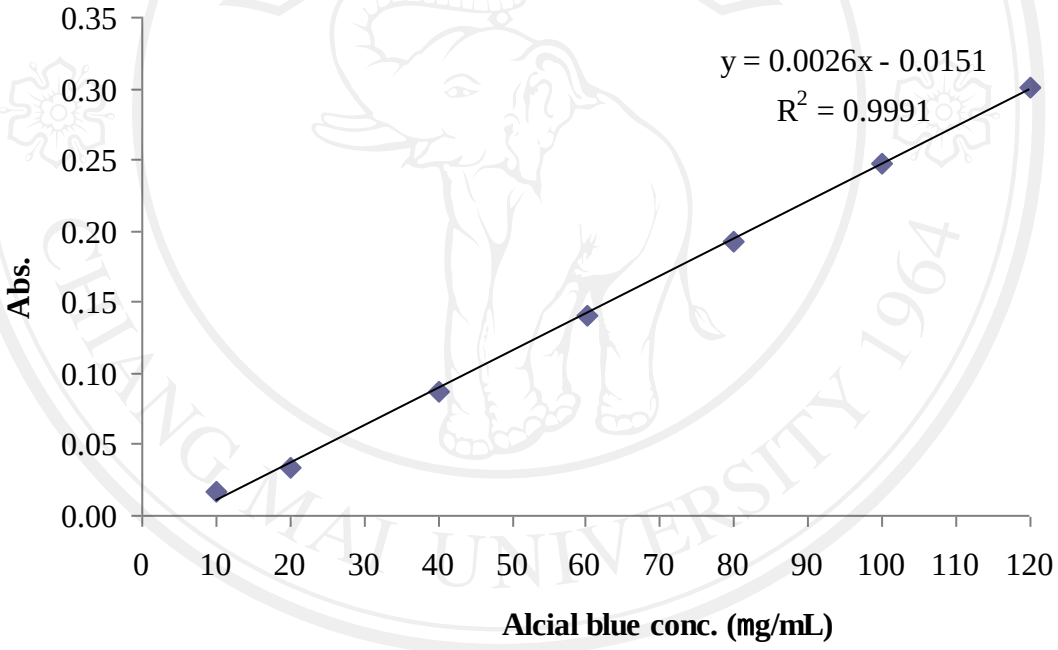


Figure 3.23 Alcian blue standard graph

Table 3.6 Effect of ML extract on gastric-wall mucus content

Group	Dose (mg/kg)	Gastric wall mucus (μg alcian blue/g wet stomach)
Normal[a]	-	25.08 ± 0.99
ML[a]	400	27.73 ± 2.53
Control[b]	-	17.10 ± 1.87*
Misoprostol[b]	0.1	32.65 ± 3.67†††
Cimetidine[b]	100	22.18 ± 3.22
ML[b]	400	33.10 ± 1.88†††

Values are expressed as mean ± S.E.M. (n=6)

a: Without gastric ulcer induction

b: Gastric ulcer induction by EtOH/HCl

* Significantly different from the normal group, $p<0.05$

††† Significantly different from the control group, $p<0.001$

3.4.6 Effect of ML extract on pylorus ligation

In this experiment, pylorus ligation caused the increase in gastric volume and total acidity and the decrease of gastric pH in the control group. Cimetidine at the dose of 100 mg/kg significantly decreased total volume, total acidity and increased pH of gastric juice ($p<0.01$) when compared with those of the control group. ML extract (400 mg/kg) showed a significant reduction of gastric volume and total acidity when compared with those of the control group. This extract tended to increase gastric pH with no statistical difference (Table 3.7).

Table 3.7 Effect of ML extract on pylorus ligation

Group	Dose (mg/kg)	Gastric volume (mL/100 g)	Gastric pH	Total acidity (mEq/L)
Control	-	2.39 ± 0.22	1.68 ± 0.10	110.1 ± 11.9
Cimetidine	100	1.39 ± 0.28**	4.6 ± 1.08**	58.5 ± 14.0**
ML	400	1.62 ± 0.24*	2.9 ± 0.84	65.0 ± 15.2*

Values are expressed as mean ± S.E.M. (n=6)

* Significantly different from the normal group, $p<0.05$

** Significantly different from the control group, $p<0.01$

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