CHAPTER IV

RESULTS

1. Primer sequences of rabbit's cytokines

Primers were designed according to the criteria as describe in the Materials and Methods using PerlPrimer version 1.1.18 and AmplifX version 1.5.4 and blasted back to Basic Local Alignment Search Tool (BLAST) N and NCBI/Primer-BLAST by using NCBI/BLAST.

The sequence of each primer was shown in Table 4. The primers for IL-10 and GAPDH had higher melting temperature than the rest. The estimation size of PCR products were ranged from 180 to 263 bp as shown in the last column of Table 4.

Table 4 Primer sequences, melting temperature, and PCR product size of rabbit's cytokines primer.

	Primer sequences	Tm	PCR product size
IL-1β	5'- CAA CAA GTG GTG TTC TCC AT-3'	44.6 °C	263 bp
	5'-GAG GTG CTG ATG TAC CAG T-3'	46.0 °C	
MCP-1	5'-CTT CTG TGC CTG CTG CTC ATA G-3'	51.6 °C	221 bp
	5'-TGC TTG GGG TCA GCA CAG AT-3'	48.7 °C	
TNF-α	5'-AGA TGG TCA CCC TCA GAT CAG -3'	49.2 °C	203 bp
	5'-GAA GAG AAC CTG GGA GTA GAT GAG -3'	52.3 °C	
TGF-β	5'-TGG ACA CCA ACT ACT GCT-3'	42.9 °C	201 bp
	5'-TGT GCT GGT TGT ACA GG-3'	41.9 °C	
IL-10	5'-GAG AAC CAC AGT CCA GCC AT-3'	62.4 °C	180 bp
	5'-CAT GGC TTT GTA GAC GCC TT-3'	60.4 °C	
GAPDH	5'-CAT CAT CCC TGC CTC CAC T-3'	62.3 °C	180 bp
	5'-GCC TGC TTC ACC ACC TTC TT-3'	62.4 °C	

2. The optimal of PCR conditions for cytokine genes

The conditions of PCR reaction were optimized by varying temperature and time of each step in PCR process: denaturation, annealing, extension, as well as concentration of primers, cDNA template, and magnesium chloride. Table 5 showed the optimal temperature and time in each step of PCR cycle.

The representative RT-PCR products from liver cDNA of high-cholesterol diet fed rabbit on an ethidium bromide-stained 1.2% agarose gel was illustrated (Figure 9). A clearly distinct band corresponding to IL-1β, MCP-1, TNF-α, IL-10, TGF-β, and GAPDH transcript would be identified in lane 2 to 7, respectively. The GAPDH was used to be the internal control and 100bp DNA ladder was used as marker.

Table 5 Specific PCR and Real-time PCR thermocycling conditions.

Step	IL-1β	MCP-1	TNF-α	IL-10	TGF-β	GAPDH
Initial denaturation	95 °C, 3 min	95 °C, 3 min	95 °C, 3 min	95 °C, 3 min	95 °C, 3 min	95 °C, 3 min
Denaturation	94 °C, 20 sec	94 °C, 20 sec	94 °C, 20 sec	94 °C, 20 sec	94 °C, 20 sec	94 °C, 20 sec
Annealing	54.5 °C, 15 sec	57.4 °C, 15 sec	56.1 °C, 15 sec	60.7 °C, 15 sec	55.1 °C, 15 sec	59.4 °C, 15 sec
Extension	72 °C, 15 sec	72 °C, 15 sec	72 °C, 15 sec	72 °C, 15 sec	72 °C, 15 sec	72 °C, 15 sec
Final extension	72 °C, 5 min	72 °C, 5 min	72 °C, 5 min	72 °C, 5 min	72 °C, 5 min	72 °C, 5 min

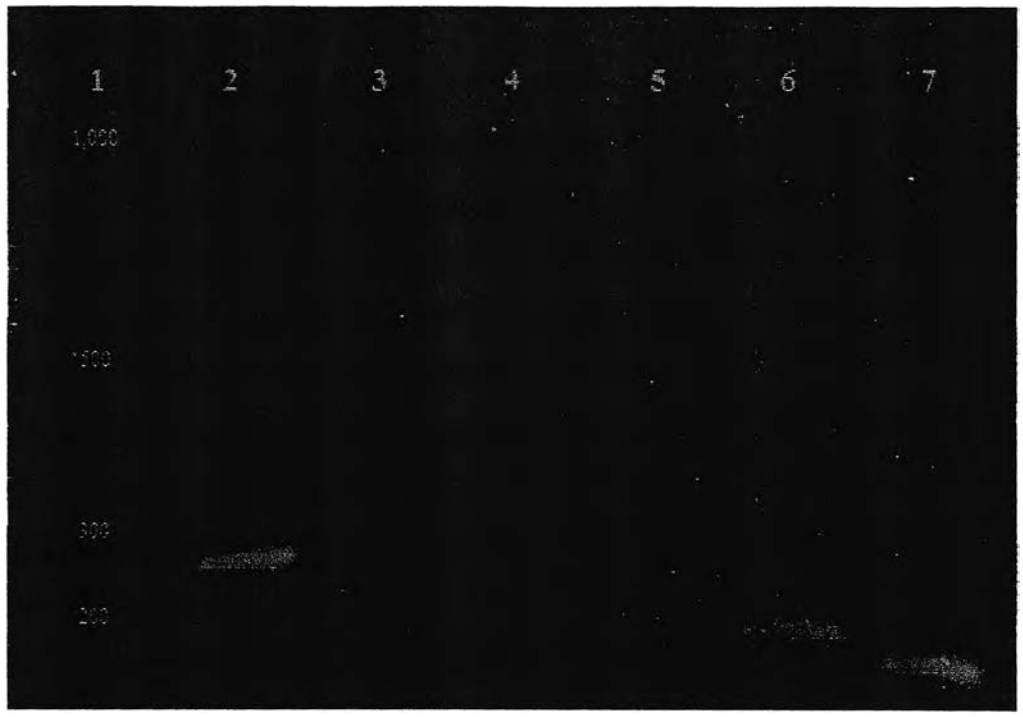

Figure 10 Agarose gel electrophoresis of RT-PCR product using liver cDNA of high-cholesterol diet fed rabbit. After amplification of PCR amplification in the presence of specific PCR primers, agarose gel containing PCR product generated from μg of cDNA was stained. Lane 1: 100bp DNA Ladder. The length of the markers in bp is shown. Lane 2: IL-1β (263 bp). Lane 3: MCP-1 (221 bp). Lane 4: TNF-α (203 bp), Lane 5: IL-10 (180 bp), Lane 6: TGF-β (201 bp), Lane 7: GAPDH (180 bp).

3. Effects of *C. comosa* on the expression of abdominal aorta cytokines

After 3 months of treatment, rabbits fed high cholesterol diet with *C. comosa* showed significant decrease of the expression of IL-1β, MCP-1, and TNF-α as compared to the rabbits fed high cholesterol diet control. The rabbits fed high cholesterol diet with simvastatin exhibited significant decrease of the expression of pro-inflammatory cytokines, IL-1β as compared to the rabbits fed high cholesterol diet control. Both *C. comosa* and simvastatin did not affect the expression of anti-inflammatory cytokines (TGF-β, IL-10) in rabbits fed high cholesterol diet as compared to rabbits fed high cholesterol diet control (Figure 10).

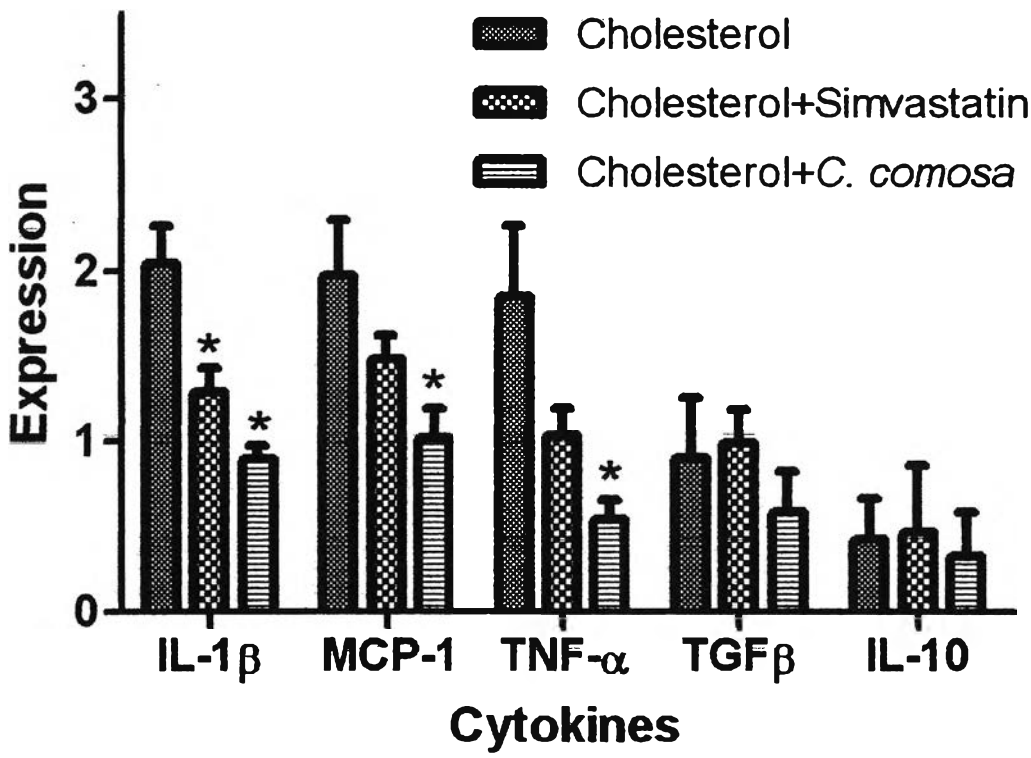

Figure 11 Effects of *C. comosa* and simvastatin on the expression of cytokines in abdominal aorta after 3 months of treatment. Rabbits were fed with high cholesterol diet, high cholesterol diet with simvastatin and high cholesterol diet with *C. comosa.* Values shown were mean ± SEM obtained from each rabbit. The experiment was performed in triplicate for 4 rabbits in each group. Significance was determined using One-way ANOVA follow by the Student-Newman-Keuls test in which p-value<0.05 was required for a statistically significant difference.

**p-value* <0.05 significant difference from high cholesterol diet control group

4. Effects of *C. comosa* on the expression of liver cytokines

After 3 months of treatment, rabbits fed high cholesterol diet with simvastatin demonstrated significant increase of the expression of the pro-inflammatory cytokines, MCP-1 and TNF-α, as compared to the rabbits fed high cholesterol diet control and the rabbits fed high cholesterol diet with *C. comosa*. In the other hand, rabbits fed high cholesterol diet with *C. comosa* exhibited significant increase of the expression of the anti-inflammatory cytokine, IL-10 as compared to the rabbits fed high cholesterol diet control (Figure 11).

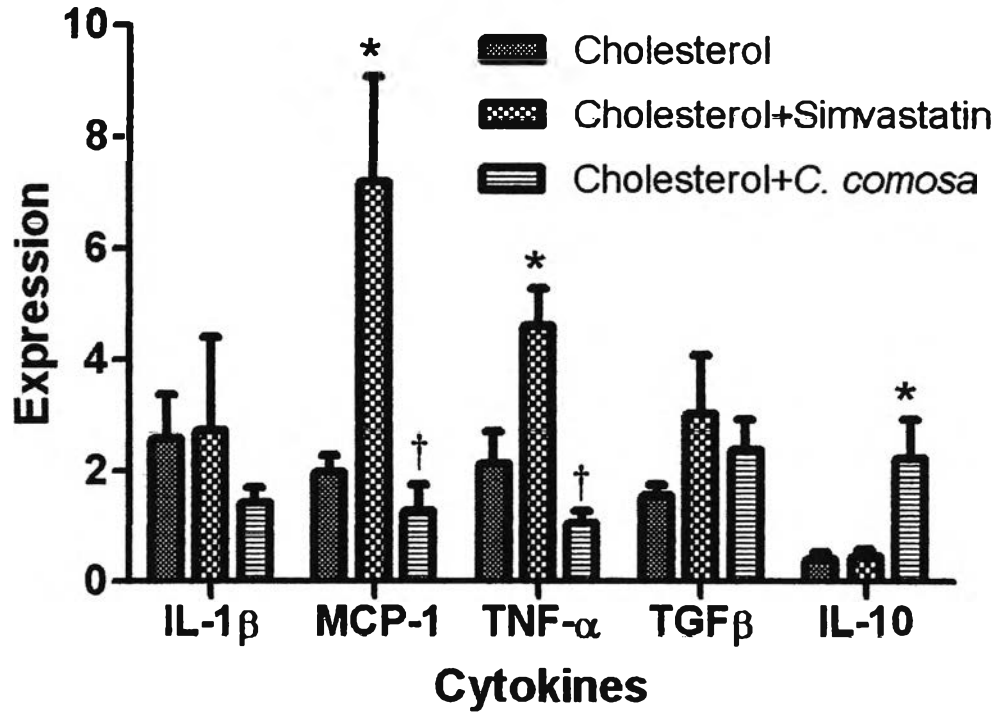

Figure 12 Effects of *C. comosa* and simvastatin on the expression of cytokines in the livers after 3 months of treatment. Rabbits were fed with high cholesterol diet, high cholesterol diet with simvastatin and high cholesterol diet with *C. comosa*. Values shown were mean ± SEM obtained from each rabbit. The experiment was performed in triplicate for 4 rabbits in each group. Significance was determined using One-way ANOVA follow by the Student-Newman-Keuls test in which *p-value*<0.05 was required for a statistically significant difference.

**p-value*<0.05 significant difference from high cholesterol diet control group

†*p-value*<0.05 significant difference between high cholesterol diet with *C. comosa* and high cholesterol diet with simvastatin group