Original article **P34**

Effect of *Curcuma comosa* on the expression of cytokine genes in rabbits fed with high cholesterol diet

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Abstract

Curcuma comosa Roxb. (Zingiberaceae), an indigenous plant in Thailand, has been used as an anti-inflammatory agent in post-partum uterine bleeding. It was shown to lower plasma lipid levels thus potentially be used in cardiovascular disease. Lipid lowering drugs like HMG-CoA reductase inhibitor including simvastatin were implicated with manifestation of liver toxicity. In the present study, we therefore investigated the possibility of liver toxicity of *C. comosa* by assessing the expression of pro-inflammatory cytokine genes as well as the serum liver enzymes in rabbits fed with high cholesterol diet combined with *C. comosa* compared to the rabbits fed with high cholesterol diet either alone or combined with simvastatin for three months. The results showed that serum liver enzymes and pro-inflammatory cytokine expression of *C. comosa* treatment group were not significantly different as compared to the high cholesterol diet control group. On the other hand, rabbits in high cholesterol diet with simvastatin treatment group significantly demonstrated an increase of alanine aminotransferase level and the expression of pro-inflammatory cytokines, MCP-1 and TNF-α as compared to the high cholesterol diet group. The pharmacological activity reported earlier and the safety regarding liver toxicity shown in this study suggested that *C. comosa* is a potentially promising candidate to be developed as an alternative agent for cardiovascular disease therapy.

Keywords: *Curcuma comosa*, liver toxicity, cytokine, IL-1, MCP-1, TNF-α

Introduction

Cardiovascular disease is the leading cause of death worldwide. Primary goal of the treatment is to prevent further morbidity and mortality from coronary heart disease (CHD) and the associated conditions. HMG-CoA reductase inhibitor is the drug of choice for lowering serum lipid in CHD treatments because of its low cost and capability to reduce the risk of ischemia in CHD patients. However, the main hindrance of drugs in this group is its liver toxicity as indicated by elevated serum alanine aminotransferase level. Therefore, efforts were tried to develop more efficient CHD drug with lower or no toxicity. *Curcuma comosa* Roxb. (Zingiberaceae) is an indigenous plant in Thailand with a common name in Thai as Wan Chak Mot Luk. It has been used traditionally as an anti-inflammatory agent in post-partum uterine bleeding. Besides the estrogenic activity, it was also found to process choleretic effect, hypocholesterolemic effect (1) and anti-atherogenic effect (2). These beneficial effects of *C. comosa* is promising to be developed as an alternative agent for CHD therapy. In addition to the pharmacological effects, safety information in term of toxicities especially liver toxicity is needed. Therefore, the aim of this study was to investigate the

possibility of liver toxicity of *C. comosa* by assessing the expression of pro-inflammatory cytokine genes as well as the serum liver enzymes in rabbits fed with high cholesterol diet combined with *C. comosa* compared to the rabbits fed with high cholesterol diet either alone or combined with simvastatin.

Materials and Methods

Materials: *C. comosa* powder was kindly provided by Professor Dr. Apichart Suksamrarn, Faculty of Sciences, Ramkamhaeng University. Simvastatin was purchased from an accredited drug store (Bangkok, Thailand).

Animals and treatment: Twelve male New Zealand white rabbits of body weight between 1.5 – 2.0 kg were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed one per cage at the Faculty of Medicine, Srinakharinwirot University, Thailand. All animals were in a controlled humidify room at a constant temperature of 25 ± 2 °C and maintained on a 12-hour alternate light-dark cycle. They were allowed to freely access to food (C.P. Company, Thailand) and drinking water. Prior to the experiment, they were randomly divided into three treatment groups of 4 rabbit each. All treatment groups were given orally with 0.1% cholesterol for 1 month. After 1 month, rabbits in group 1, 2, 3 were given orally for 3 months with 0.5% cholesterol, 0.5% cholesterol combined simvastatin at the dosage of 5 mg/day and 0.5% cholesterol combined *C. comosa* at the dosage of 400 mg/kg/day, respectively.

Blood and liver sample collection: Blood samples were collected from 12 hours fasted rabbit at the end of treatment. Plasma were separated and analyzed for liver function parameters (alanine aminotransferase, ALT; aspartate aminotransferase, AST; alkaline phosphatase, ALP) using auto-analyzer (Hitachi 917) at Professsional Laboratory Management Corp Co., Ltd., Bangkok. Liver samples were kept at -80 °C until assay.

RNA extraction and reverse transcription: RNA was extracted using Trizol® reagent according to the manufacturer's instruction (Invitrogen, USA). RNA quantity was measured by spectrophotometer at 260 nm absorbance and stored at -80 °C. Process of reverse transcription was described in RT script kit's manual protocol (USB, USA). cDNA was synthesized by MMLV-reverse transcriptase and using random hexamer as probe. The condition for reverse transcription was 42 °C for an hour.

Real-time RT PCR: The expression of cytokine genes analysis was performed with iQ™ multicolor real-time PCR detector system and iQ™ SYBR® Green Supermix as recommended by the manufacturer. The cDNA templates were probed with the specific primers (Table 1) designed by PerlPrimer version 1.1.18 for IL-1 (Accession No M26295.1), MCP-1 (Accession No NM_001082294.1) and TNF-α (Accession No NM_001082263.1) while GAPDH (Accession No NM_001082253.1), an internal control gene, was used as previously reported (3). Amplification was performed under the condition using thermocycler for 40 cycles at 95 °C 30 seconds, specific annealing temperature (Table 1) 30 seconds and 72 °C 30 seconds. The calculation of the Threshold cycle (Ct) was performed using iQ™ optical system software version 2.0. Amplification's specificity was verified by melt curve analysis and gel electrophoresis.

Statistical analysis: The data were analyzed by using one-way analysis of variance (one-way ANOVA) calculated from SPSS version 16.0 software program (SPSS Inc., USA). The significant different between group is at the level of p-value <0.05.

Table 1 Primer and specific annealing temperature for PCR amplification

Cytokines	Primer sequences	Annealing temperature
IL-1	sense: 5'-CAA CAA GTG GTG TTC TCC AT-3' anti-sense: 5'-GAG GTG CTG ATG TAC CAGT-3'	55.0 °c
MCP-1	sense: 5'-CTT CTG TGC CTG CTG CTC ATA G-3' anti-sense: 5'-TGC TTG GGG TCA GCA CAG AT-3'	57.1 °c
TNF-α	sense: 5'-AGA TGG TCA CCC TCA GAT CAG-3' anti-sense: 5'-GAA GAG AAC CTG GGA GTA GAT GAG-3'	61.4 °c
GAPDH	sense: 5'-CAT CAT CCC TGC CTC CAC T-3' anti-sense: 5'-GCC TGC TTCA CCA CCT TCT T-3'	65.0 °c

Figure 1 showed that rabbit-fed high cholesterol diet with simvastatin significantly increased the expression of MCP-1 and TNF-α as compared to the rabbit-fed high cholesterol control and the rabbit-fed high cholesterol diet with *C. comosa*. The rabbit-fed high cholesterol diet with *C. comosa* did not affect all pro-inflammatory cytokines when compared to the rabbit-fed high cholesterol control.

Table 2 Liver functions at 4 months of rabbits

Groups	Liver functions		
	AST (U/L)	ALT (U/L)	ALP (U/L)
0.5% cholesterol	51.00 ± 8.93	34.00 ± 4.76	65.75 ± 14.59
0.5% cholesterol + 5 mg/day simvastatin	73.00 ± 7.70	102.50 ± 26.21*	81.25 ± 19.29
0.5% cholesterol + 400 mg/kg/day *C. comosa*	53.75 ± 4.97	28.00 + 1.00	47.75 ± 3.25

Value were shown as the mean ± SEM (n = 4). *p<0.05 significant difference from high cholesterol-fed control group. ALT = alanine aminotransferase, AST = aspartate amino transferase, ALP = alkaline phosphatase.

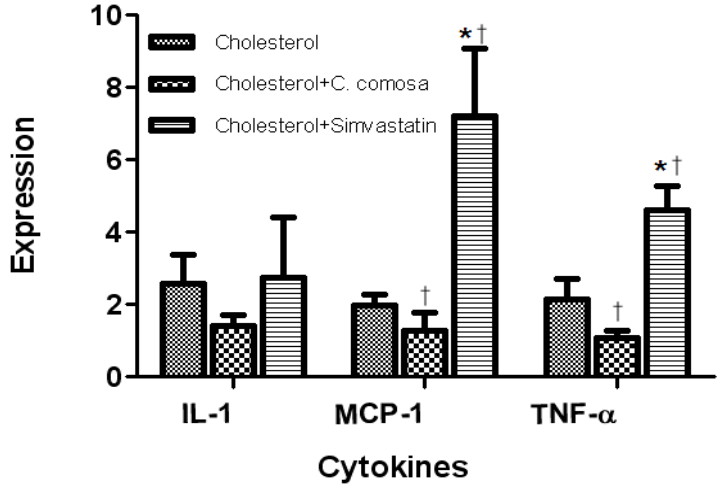

Figure 1 Effect of *C. comosa* on the expression of cytokine genes

Data were shown as the mean ± SEM (n = 4). *p<0.05 significantly different from high cholesterol diet fed control group.
† p<0.05 high cholesterol-diet with *C. comosa* vs high cholesterol-diet with simvastatin.

Discussion

Among the lipid lowering drugs used in Thai patients in term of cost-effectiveness, simvastatin is the most popular as compared to atorvastatin and rosuvastatin (4). Simvastatin not only reduces LDL cholesterol but also possesses pleiotropic effect on other cellular functions including anti-inflammatory effect and anti-oxidation. These effects not only provide the advantageous effects on blood vessel but the pleiotropic effect can also induce liver injury by inhibition of selenoproteins (5). Most cases with liver injury were associated with an increase of serum ALT (6). Rabbit-fed high cholesterol diet with *C. comosa* at 400

mg/kg/day for 3 months was previously found to reduce plasma LDL-cholesterol (7). Serum and liver samples from these groups of animals were used in this study to investigate the toxicity of *C. comosa* and simvastatin in the livers. The results from this study showed that serum ALT was significantly increased in rabbit-fed high cholesterol diet with simvastatin indicating the liver toxicity as previously seen in human (8) while *C. comosa* did not affect ALT level.

To further investigate the cytokine gene expression to explain the liver toxicity data for *C. comosa* and simvastatin, in this study we found that simvastatin treatment induced pro-inflammatory cytokines, MCP-1 and TNF-α, gene expression while *C. comosa* did not. This implied that there was a direct injury on hepatocyte to induce cytokines expression and thus overcome the anti-inflammatory effect of simvastatin. In contrast, *C. comosa* treatement did not significantly increase serum liver enzymes. Consistently, pro-inflammatory cytokine expression did not significantly increase but tend to diminish instead.

Conclusion

In conclusion, *C. comosa* treatment at 400 mg/kg/day in rabbits fed with high cholesterol diet did not change serum liver enzymes after three months of the treatment. Consistently, the expression of pro-inflammatory cytokines including IL-1, MCP-1 and TNF-α were not affected. These findings may provide the possibility that *C. comosa* is a promising candidate to be developed for coronary heart disease therapy without liver toxicity. However, the benefits and toxicities of *C. comosa* require further experimental and clinical studies.

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