



Original Article

## The effects of the extracts from *Carthamus tinctorius* L. on gene expression related to cholesterol metabolism in rats

Teerakul Arpornsuwan<sup>\*1</sup>, Khaimuk Changsri<sup>1</sup>, Sittiruk Roytrakul<sup>2</sup> and Tadsanee Punjanon<sup>3</sup>

<sup>1</sup> Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Khlong Luang, Pathum Thani, 12120 Thailand.

<sup>2</sup> Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology, Thailand Science Park, Klong Luang, Pathum Thani, 12120 Thailand.

<sup>3</sup> Pharmacology and Toxicology Unit, Department of Medical Sciences, Faculty of Science, Rangsit University, Khlong Luang, Pathum Thani, 12000 Thailand.

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### Abstract

*Carthamus tinctorius* L. (safflower) is in Thailand traditionally used for a herbal tea for health to reduce cholesterol and prevent atherosclerosis. The present study was aimed to investigate the effect of the crude extracts from safflower on cholesterol metabolism in high cholesterol fed rats. The crude extract was fractionated in hexane, dichloromethane, and methanol. To evaluate the hypolipidemic effect, the safflower extracts were daily fed to normal and hyperlipidemic rats induced by 2%-cholesterol diet (W/W) supplementation, at dose of 250 mg/kg body wt. During the 4-week study, body weight, food intake, organ weight, and plasma cholesterol levels were evaluated. Animals treated with 2%-cholesterol diet and dichloromethane fraction for a week exhibited decreased body weight. After treatment for 14 and 30 days, a significant reduction in total cholesterol and total cholesterol/HDL-cholesterol and a significant induction in HDL-cholesterol were observed in the hypercholesterolemic rats treated with the dichloromethane extract. Higher expression of SRBI and ABCA1 in the liver of the control group was observed after 4 weeks whereas no significant difference in the expression level of SRBI and ABCA1 was found in groups treated with extract after 2 and 4 weeks. The results of this study suggested that the dichloromethane extract can reduce the total cholesterol/HDL-cholesterol of hyperlipidemic rats. The expression of SRBI and ABCA1 mRNA may not be regulated by the crude extract of safflower, which may not in part explain the decrease in HDL-cholesterol and gene encoding enzymes of the cholesterol biosynthetic pathway.

**Keywords:** *Carthamus tinctorius* L, dichloromethane fraction, scavenger receptor class B type 1, ATP binding cassette, subfamily A member 1, hypolipidemic effect

### 1. Introduction

Safflower (*Carthamus tinctorius* L.) is a member of the family Compositae or Asteraceae. It is cultivated mainly

for its seed, which are used for making edible oil and as birdseed. In Thailand, safflower has been use as herbal tea for health. There are records that it is used for reducing ailments from the neurotropic, cardiotropic, hemopoietic, and diaphoretic systems. Many clinical and laboratory studies support the use of the medicine properties of safflower for menstrual problems, cardiovascular disease, pain, and swelling associated with trauma (Punjanon *et al.*, 2004).

\* Corresponding author.  
Email address: [teerakul@tu.ac.th](mailto:teerakul@tu.ac.th)

Safflower seeds are rich in edible oil, with oil content similar to olive, sunflower, and peanut oils (40% dry matter weight). Besides, this oil is composed of 75% linoleic acid (C18:2). It is well known that dietary phospholipids effectively lower the serum cholesterol level, although the mechanisms underlying the hypocholesterolemic effects have not been clarified. The feeding of purified or crude safflower phospholipids resulted in some desirable alterations, such as the reduction of plasma and liver lipid levels, an increase in high density lipoprotein-cholesterol (HDL-C), and the excretion of fecal neutral steroid (Iwata *et al.*, 1991; 1992). Previous studies reported that feeding of diets containing safflower phospholipids (crude phospholipid or purified phospholipids) to laying hens resulted in a decrease in liver triglycerides and serum cholesterol without adverse effects (An *et al.*, 1997). Moreover, the effects of defatted safflower seed extract and phenolic compounds in diet on plasma and liver lipid were investigated in ovariectomized rats fed with high-cholesterol diets (Cho *et al.*, 2004). The safflower polyphenols have the effect of improving blood lipid status via increasing HDL-cholesterol formation and cholesterol excretion without significant uterotrophic action in estrogen-deficient animals (Cho *et al.*, 2004).

Plasma HDL plays a critical role in cholesterol metabolism *in vivo*. HDL particles mediate the transport of cholesterol from peripheral tissues to the liver in a process termed reverse cholesterol transport, which is believed to play a critical role in whole-body cholesterol homeostasis (Johnson *et al.*, 1991). The ATP binding cassette, subfamily A, member 1 (ABCA1) mediates the rate-controlling step in HDL particles formation by promoting the efflux of cholesterol and phospholipids to apoA-I (Schmitz and Langmann, 2001). Over expression of hepatic ABCA1 raises HDL cholesterol levels (Basso *et al.*, 2003; Wellington *et al.*, 2003), and liver-specific deletion of ABCA1 results in a substantial decrease in plasma HDL cholesterol in chow-fed mice (Timmins *et al.*, 2005).

The scavenger receptor class B, type I (SRBI) is most abundantly expressed in the liver and steroidogenic tissues, which are the most active sites of HDL selective lipid uptake (Acton *et al.*, 1996). Hepatic over expression of murine SR-BI in mice substantially reduces plasma HDL and increases biliary cholesterol (Kozarsky *et al.*, 1997). There is no study that correlates the effect of crude extract of the flower of safflower on lowering plasma cholesterol with the expression of ABCA1 and SRBI. The current study was designed to investigate the effectiveness of the crude extract of *Carthamus tinctorius* flower in treating the hypercholesterolemic phenotype in the rats fed with high cholesterol diets. Because of the similarities in the mechanism of lower blood cholesterol, we utilized atorvastatin as a reference drug. Physiological parameters such as changes in body weight, organ weight, plasma cholesterol, and plasma triglyceride levels were evaluated. In addition to physiological effects, the gene known to be associated with cholesterol metabolism were measured for change in their transcripts abundance relative

to a control diet in order to better understand the anti-cholesterol effect of safflower extracts.

## 2. Material and Methods

### 2.1 Preparation of the crude extract of *C. tinctorius* L.

*C. tinctorius* L. was grown at the Department of Agricultural Extension, Ministry of Agricultural and Cooperatives, Thailand. Dried petals of *C. tinctorius* flowers (1.55 kg) were extracted with hexane using the Soxhlet. The precipitable fraction was fractionated into dichloromethane and methanol. Yields of the extracts obtained from hexane, dichloromethane and methanol were 4.91, 3.57, and 5.31% (w/w), respectively.

### 2.2 Animals and diets

Adult male rats (Wistar-strain, 8 weeks old) were purchased from the National Animal Institute of Thailand. Animals were individually housed in stainless steel cages in a room at 23-25°C under 12-hour cycles of light and darkness. The rats were randomly assigned to six diet groups with 5-10 rats in each group. These rats were fed with one of the following diets with or without safflower extract (250 mg/kg body wt.): (1) normal rodent chow diet, served as untreated "control" group ( $n = 10$ ), (2) control diet supplemented with 2%-cholesterol, "Chol" group ( $n = 10$ ), (3) high cholesterol diet supplemented with hexane fraction, "Chol+SFh" group ( $n = 10$ ), (4) high cholesterol diet supplemented with dichloromethane fraction, "Chol+SFd" group ( $n = 5$ ), (5) high cholesterol diet supplemented with methanol fraction, "Chol+SFm" group ( $n = 5$ ), and (6) high cholesterol diet supplemented with atorvastatin, "Chol+atorvastatin" (75 mg/kg). All animals were housed separately and allowed free access to food and tap water. Body weight and food intake were recorded weekly. Animal care and handling was conformed to accepted guidelines (Guide for the care and use of laboratory animals Washington DC, National Institute of Health; 1985).

After 2 and 4 weeks, animals were anesthetized with ether and killed following a 12 hrs fasting. Blood was collected, and placed on ice. Plasma was obtained by centrifuging the blood at 1000 X g for 15 min and stored at -20°C for lipid analyses. Liver and kidneys were excised and weighed. Parts of the liver were snap-frozen in liquid nitrogen and stored at -70°C for total RNA isolation. Total cholesterol and lipid profile measurements were carried out by Dimension RxL (Dade Behring) in Thammasat Chalerm Prakiet Hospital using enzymatic assay reagent kits obtained from Dade Behring (Thailand).

### 2.3 Determination of ABCA1 and SRBI gene expression by RT-PCR

Total RNA from different group of rat liver and the cell pellet was isolated as described by Chang *et al.* (1993)

with some modification. RNA samples were quantified by reading the optical density at a wavelength of 260 nm. Total RNA from control or treated groups was reverse transcribed using oligo dT 20 primer and ImProm-II Reverse transcription system (Promega Corporation). The synthesis of the first-strand cDNA was run at 42°C for 60 min. The reaction mixture was then subjected to incubate at 70°C for 15 minutes in order to inactivate the enzyme. PCR was carried out in a total volume of 25 µl using Go Taq Green master mix (Promega Corporation) containing 1.5 mM MgCl<sub>2</sub>. The reaction mixture was subjected to 30 rounds of PCR amplification. The sequence of primers used was as follows: 5'-GGGATGTGG AAGGAGATCCC-3' (forward) and 5'-CCCAAGGTCATTA GCAGCTTCA-3' (reverse) for SRB1 (accession no. NM\_016741); 5'-CAGGAGGTGATGTTTCTGACCA-3' (forward) and 5'-CATTGCCTCTGAGGTCCAAC-3' (reverse) for ABCA1 (accession no. NM\_178095) and 5'-TTGTAACCAA CTGGGACGATATGG-3' (forward) and 5'-GATCTTGATCTT CATGGTGCTAGG-3' (reverse) for β-actin (accession no. V01217). The PCR profile consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 45 sec, 45 sec of annealing at 50°C and extension at 72°C for 45 sec. A final extension cycle at 72°C for 10 min was completed before the reaction terminated by lowering the temperature to 4°C. The PCR product was analyzed by 1.2%-agarose gel electrophoresis to allow separation of the amplified fragments. The gel was then stained in 1 µg/ml ethidium bromide for 10 min, followed by destaining in water for 10 min. Bands was then visualized under short wavelength UV light. To correct for possible difference in RNA concentration used, all experimental data were compensated with rodent β-actin intensity as an internal standard.

## 2.4 Statistics

All results are expressed as mean ± SEM (standard error of the mean) unless stated otherwise. The paired Student's test was used to evaluate the statistical significance of differences between paired observations. A value of  $p < 0.05$  was considered to be significant in all cases.

## 3. Results

### 3.1 Body weight and organ weight

Wistar-strain rats fed 2%-cholesterol chow diet did not exhibit an increased body weight over control. Feeding with hexane fraction and methanol fraction showed no significant difference with 2%-cholesterol chow diet but dichloromethane fraction significantly decreased the body weight versus the control diet and the high cholesterol diet ( $p \leq 0.05$ ) at week 1 (Table 1). Similar results were remained when compare to the control group continue feeding with a normal and 2%-cholesterol chow diet ( $p \leq 0.05$ ) at week 2, 3, and 4.

Table 2 shows the liver weights of the Wistar-strain rats fed 2%-cholesterol chow diet supplemented with hexane, dichloromethane, methanol fraction or atorvastatin. The liver weights of the rats fed 2%-cholesterol and supplemented with hexane, dichloromethane and methanol fraction, and with atorvastatin were significantly higher than the control rats fed a normal diet ( $p \leq 0.05$ ) at week 2. At week 4 the liver weights of all groups were not significantly different from those of the control group fed a normal diet. The kidney weights of all groups fed 2%-cholesterol chow diet with and without fractions of *C. tinctorius* were not significantly different from those of the control group fed a normal diet at week 2 and 4.

Table 1. Effects of three different fractions of *C. tinctorius* supplementation on weight gain in rats fed normal and 2%-cholesterol diet.

group	Weight gain (g)				
	week 0(n=10)	week 1(n=10)	week 2(n=10)	week 3(n=5)	week 4(n=5)
Fed with normal diet, ND					
ND	181.4±12.2	227.0±14.6	281.6±15.2	326.0±28.6	351.0±38.9
Fed with 2%-cholesterol (2%-chol)					
2%-Chol	190.6±8.3	233.2±14.1	279.6±18.6	323.6±24.7	354.0±31.4
2%-Chol+SFh	190.2±4.0	230.2±7.3	273.0±9.7	314.0±13.5	334.8±19.1
2%-Chol+SFd	187.0±6.9	216.2±8.2 <sup>b</sup>	259.6±13.5 <sup>a,b</sup>	292.4±12.6 <sup>a,b</sup>	310.8±14.9 <sup>a,b</sup>
2%-Chol+SFm	196.2±7.5	238.0±11.1	285.0±12.6	324.4±16.5	347.2±17.8
2%-Chol+ atorvastatin	181.8±11.4	222.6±13.5	271.4±16.0	313.2±11.5	341.2±13.3

Values are shown in mean ± SD (n=5).

<sup>a</sup> Significantly different from those of the control group fed with normal diet at  $p \leq 0.05$ .

<sup>b</sup> Significantly different from those of the group fed with 2% cholesterol diet at  $p \leq 0.05$ .

SFh, SFd, SFm: three different fractions of *C. tinctorius* from hexane (SFh), dichloromethane (SFd), and methanol (SFm).

Table 2. Effects of three different fractions of *C. tinctorius* supplementation on organ weights in rats fed normal and 2%-cholesterol diet

group	Organ weights (gram)			
	Liver weight		Kidney weight	
	week 2	week 4	week 2	week 4
Fed with normal diet (ND)				
ND	9.9±1.12	12.3±1.76	2.0±0.16	2.2±0.17
Fed with 2%-cholesterol (2%-chol)				
2%-Chol	10.5±1.32	12.0±0.89	2.0±0.22	2.2±0.23
2%-Chol+SFh	11.7±0.53 <sup>a</sup>	12.7±0.85	2.0±0.08	2.3±0.27
2%-Chol+SFd	11.6±0.86 <sup>a</sup>	11.4±0.71	2.0±0.30	2.1±0.15
2%-Chol+SFm	11.4±0.30 <sup>a</sup>	13.2±1.04	1.9±0.14	2.2±0.11
2%-Chol+ atorvastatin	12.1±0.89 <sup>a</sup>	12.1±0.83	1.9±0.12	2.1±0.12

Values are shown in mean ± SD (n= 5).

<sup>a</sup>Significantly different from those of the control group fed with normal diet at  $p \leq 0.05$ .

SFh, SFd, SFm: three different fractions of *C. tinctorius* from hexane (SFh), dichloromethane (SFd), and methanol (SFm).

### 3.2 Lipid profile

Rats fed the high cholesterol diets displayed a significant ( $p \leq 0.05$ ) increase in the total plasma cholesterol and the ratio of total cholesterol to HDL-C throughout the study (Figure 1A, 3). No differences were observed in plasma LDL-cholesterol and triglyceride between the control and high cholesterol diets group (Figure 1B, 2B). The plasma HDL-cholesterol decreased in hypercholesterolemia rats induced by high cholesterol diets (Figure 2A). The total plasma cholesterol in rats fed the 2%-cholesterol supplemented with the dichloromethane fraction was significantly lower than the control group fed the 2%-cholesterol diet ( $p \leq 0.05$ ) at week 4 (Figure 1A). Moreover, the plasma HDL-C of rats fed dichloromethane fraction was significantly higher at week 4 ( $p \leq 0.05$ ) than the 2%-cholesterol diet and the ratio of total cholesterol to HDL-C was significantly lower than rats fed the 2%-cholesterol diet ( $p \leq 0.05$ ) at week 2 and 4 (Figure 2A, 3). The plasma level of LDL-cholesterol of the positive control group fed the 2%-cholesterol and atorvastatin was higher than those of the control rats fed with either a normal diet or 2%-cholesterol diet ( $p \leq 0.05$ ) at week 4 (Figure 2B). The ratio of total cholesterol to HDL-C in the positive control rats fed the 2%-cholesterol containing atorvastatin was significantly lower than the control rats fed a 2%-cholesterol diet ( $p \leq 0.05$ ) (Figure 3). From the results above the dichloromethane supplement is capable to lower the cholesterol in rats as well as the atorvastatin (positive control).

### 3.3 mRNA expressions of ABCA1 and SRBI in rat livers

The effects of three fractions of *C. tinctorius* on the expression of ABCA1 and SRBI from liver rats fed the 2%-

cholesterol diet for 2 and 4 weeks are presented in Figure 4. The levels of ABCA1 in the livers of rats fed a normal diet and the 2%-cholesterol diet were not detectable at week 2. Atorvastatin diets showed a trend towards an increase in the expression of ABCA1 in hypercholesterolemic rat livers. Similar increase in the level of ABCA1 transcript was observed in livers from rats supplemented with hexane, dichloromethane, and methanol fraction (Figure 4A).

As shown in Figure 4B, the levels of SRBI in the livers of the rats fed a normal diet and 2%-cholesterol diet were not detectable at week 2. The Wistar-strain rats fed *C. tinctorius* fractions showed a marked increase in hepatic SRBI expression as similar to the control group. Whereas the increase in the SRBI content in the control group fed with normal, 2%-cholesterol diet, hexane, dichloromethane, and methanol fractions supplemented group was observed at week 4.

## 4. Discussion

The current study demonstrated the effect of *C. tinctorius* flower extracts in treating the hypercholesterolemic phenotype in the Wistar-strain rat model. When comparing the effectiveness of *C. tinctorius* flower extract to the anti-cholesterol drug, atorvastatin, we observed similar, yet distinct, therapeutic effects of safflower. Transcription abundance of genes known to be involved in the cholesterol metabolism response was also determined as a measure of their expression in order to help elucidate their potential roles in the physiological effects of safflower.

Several *in vivo* studies have observed that a high fat diet rich in linoleic acid (safflower oil diet) induced a significant increase in epididymal and perirenal adipose tissues in

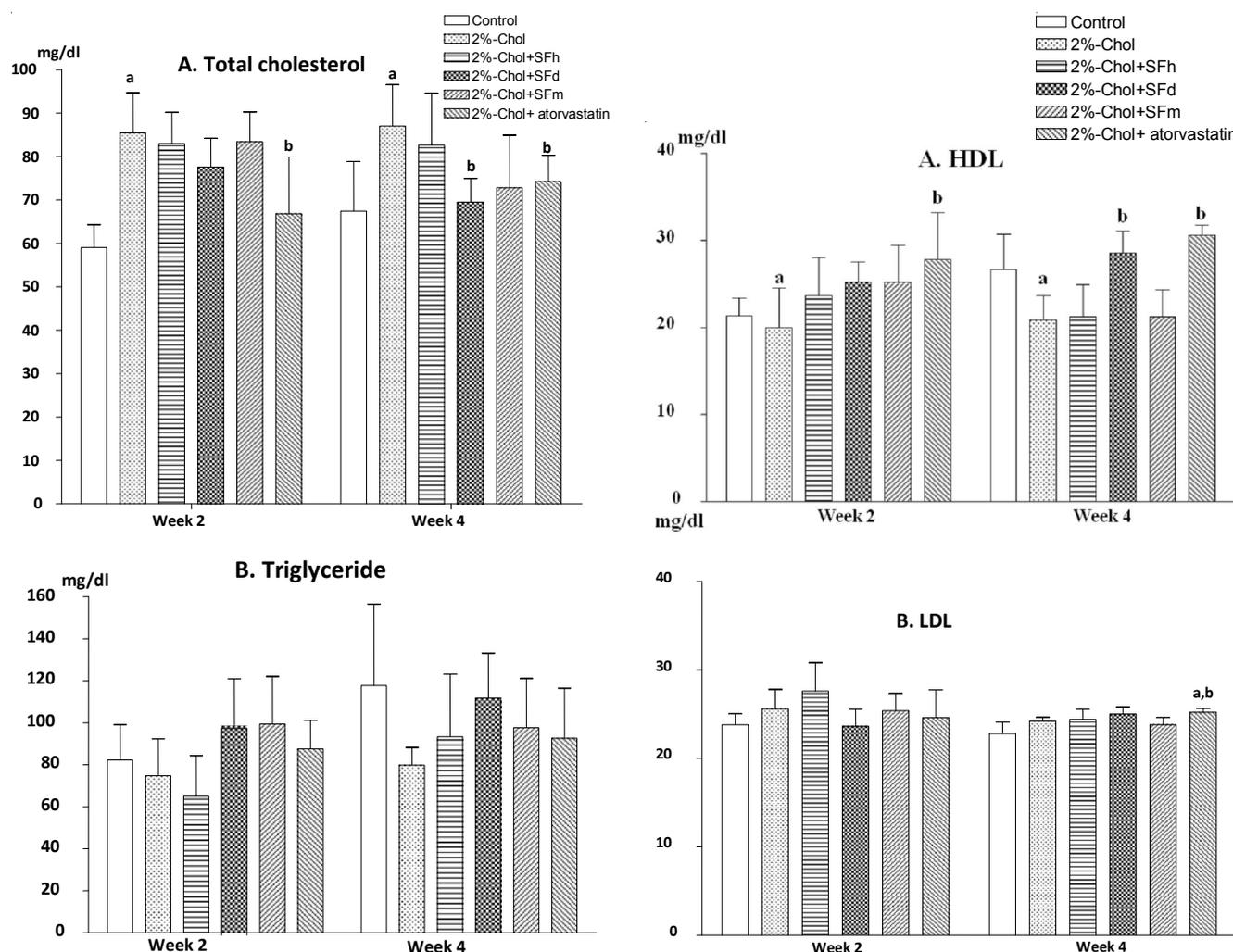


Figure 1. Effect of three different fractions of *C. tinctorius* supplementation on plasma total cholesterol (A) and triglyceride (B) in rats fed normal and 2%-cholesterol diet. Values are shown in mean  $\pm$  SD (n=5). SFh, SFd, SFm: three different fractions of *C. tinctorius* from hexane (SFh), dichloromethane (SFd) and methanol (SFm).

<sup>a</sup> Significantly different from those of the control group fed with normal diet at  $p \leq 0.05$ .

<sup>b</sup> Significantly different from those of the group fed with 2%-cholesterol diet at  $p \leq 0.05$ .

comparison with low-fat diet, while other high-fat diets rich in gamma-linolenic acid or in omega-3 fatty acids failed to do (Takahashi and Ide 2000; Takahashi *et al.*, 2000). In the current study, the Wistar-strain rats consumed comparable amount of food and water among all groups (data not shown). Body weights of all rats fed a normal diet or the 2%-cholesterol were comparable (Table 1).

The liver weights of the rats fed the 2%-cholesterol chow diet were significantly increased compared to the rats fed a normal diet (Table 2). It has been shown that dietary conjugated linoleic acid (CLA) feeding resulted in the

Figure 2. The effect of three different fractions of *C. tinctorius* supplementation on plasma HDL-cholesterol (A) and LDL-cholesterol (B) in rats fed normal and 2%-cholesterol diet.

Values are shown in mean  $\pm$  SD (n=5).

SFh, SFd, SFm: three different fractions of *C. tinctorius* from hexane (SFh), dichloromethane (SFd), and methanol (SFm).

<sup>a</sup> Significantly different from those of the control group fed with normal diet at  $p \leq 0.05$ .

<sup>b</sup> Significantly different from those of the group fed with 2%-cholesterol diet at  $p \leq 0.05$ .

development of hepatomegaly and fatty liver in mice (Takahashi *et al.*, 2003) and a mixture of CLA also induced hepatic lipid accumulation in rat. In addition, higher total cholesterol and ratio of total cholesterol to HDL were observed in rats fed the 2%-cholesterol chow diet (Figure 1A, 3). These results suggested that the feeding of high fat-diets is associated, at least in part, with increases in plasma lipid content.

In Table 1 and Figure 1A, 3 the body weight and the

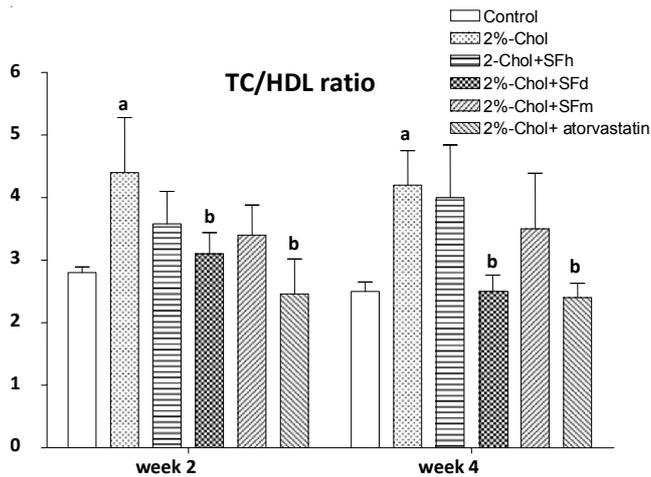


Figure 3. The effect of three different fractions of *C. tinctorius* supplementation on the ratio of total cholesterol to HDL of rats fed normal and 2%-cholesterol diet. Values are shown in mean  $\pm$  SD (n=5). SFh, SFd, SFm: three different fractions of *C. tinctorius* from hexane (SFh), dichloromethane (SFd), and methanol (SFm).

<sup>a</sup> Significantly different from those of the control group fed with normal diet at  $p \leq 0.05$ .

<sup>b</sup> Significantly different from those of the group fed with 2%- cholesterol diet at  $p \leq 0.05$ .

plasma lipid profile, the total cholesterol and the ratio of total cholesterol to HDL of rats fed the 2%-cholesterol diet supplemented with dichloromethane fraction for 4 weeks were significantly decreased. The liver weights of the rats fed the 2%-cholesterol chow diet in the presence or absence of hexane, dichloromethane or methanol fraction were comparable to the rats fed a normal diet (Table 2). There was also no significant difference between groups in the kidney weights at week 4 of all groups fed the 2%-cholesterol chow diet with and without fractions of *C. tinctorius* and those of the control group fed with a normal diet. Taken together, the significant lower body weight of the dichloromethane group coincided with a decrease in the total cholesterol and an increase in the HDL-cholesterol when compared to the control. Also to note, the dichloromethane and atorvastatin group showed no differences in plasma triglyceride. This may imply that dichloromethane fraction alters cholesterol metabolism but not triglyceride metabolism via similar mechanism as atorvastatin.

The results of this study demonstrated that the mRNA expression of ABCA1 and SRBI in the liver tended to increase with dietary fat intake in Wistar-strain rats (Figure 4). The plasma cholesterol and the ratio of total cholesterol to HDL are positively correlated to the expression of HDL metabolism (Figure 1, 3). The high fat diets used in this study presumably provide more unsaturated fatty acids resulting in metabolic changes of HDL metabolism. After 2 weeks, a

significant higher up-regulation of ABCA1 and SRBI mRNA expressions in rats fed the 2%-cholesterol chow diet containing hexane, dichloromethane, methanol fractions, or atorvastatin were observed when compared to those fed a normal and the 2%-cholesterol chow diet. However, the ratio of total cholesterol to HDL-C was significantly lower in the dichloromethane fraction supplemented group than in 2%-cholesterol diet group ( $p \leq 0.05$ ) at 2 and 4 week (Figure 3). ABCA1 is particularly abundant in the liver, and the appropriate regulation of ABCA1 is critical for a selective increase in HDL cholesterol levels (Wellington *et al.*, 2003). ABCA1 will elevate plasma HDL cholesterol levels and decrease atherosclerosis (Clee, 2000). In this study, administration of the dichloromethane fraction or atorvastatin, however, could up-regulate the expression of ABCA1 and increase the plasma HDL-C. It suggested that dichloromethane may play a regulation role on plasma HDL-C via ABCA1 expression.

From results in Table 2 and Figure 4, the animals fed the atorvastatin and the safflower extract diet, exhibited an increase in SRBI expression level versus the control and high cholesterol fed rodents at the first 2 weeks. The rats fed normal diet and 3 fractions of safflower demonstrated an increase in liver weight and SRBI expression versus the normal fed rats. The dichloromethane fraction induced lipid-lowering effect that we observed was limited to cholesterol similar to atorvastatin treatment. This observation supports the proposition that SRBI may involve in the regulation of lipid metabolism by dichloromethane fraction.

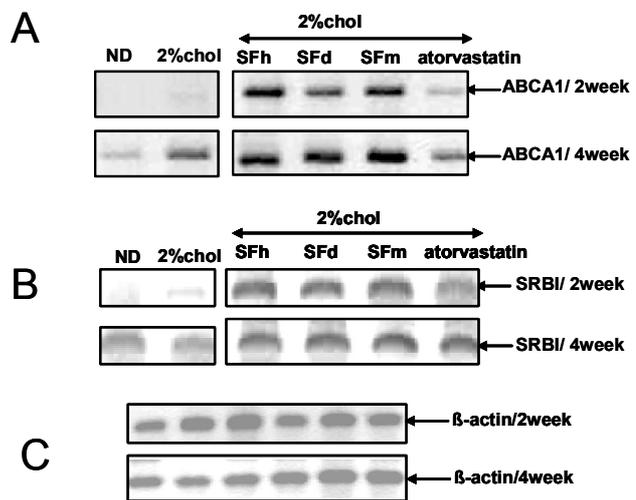


Figure 4. Effects of three different fractions of *C. tinctorius* supplementation on mRNA expression of ABCA1 and SRBI in rat livers after 2 and 4 weeks. The mRNA expression of rat ABCA1 (A) and SRBI (B) were measured by semi-quantitative RT-PCR as described in the method. (C) All experimental data were compensated with rodent  $\beta$ -actin intensity as an internal standard. Images are representative of three different experiments with triplicate in each.

## 5. Conclusion

*Carthamus tinctorius* dichloromethane extract modified the hypercholesterolemic phenotype and genes associated with cholesterol metabolism in the Wistar strain rats. Rats that fed a high-cholesterol diet and got orally administered dichloromethane extract of *C. tinctorius* flower showed lower body weight and total plasma cholesterol. The metabolic changes in hypercholesterolemic rats caused by an intake of anti-cholesterol drug atorvastatin and safflower dichloromethane fraction resulting in the HDL-cholesterol increasing effect and total cholesterol decreasing effect may be linked to the up-regulation of ABCA1 and SRB1 mRNA in the liver. This is paradoxical to the proposal role of safflower dichloromethane fraction in the regulation of cholesterol metabolism but not triglyceride metabolism in rats.

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