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Evaluation of the Efficacy of *Kochiae fructus* Extract in the Alleviation of Carbon Tetrachloride-induced Hepatotoxicity in Rats*

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Hepatoprotective effects of the extract of *Kochiae fructus* (KF), a traditional oriental medicinal plant, were evaluated against carbon tetrachloride (CCl_4)-induced liver damage in rats. Male Sprague-Dawley rats were divided into control, CCl_4, CCl_4 plus methanol extract of KF (KFM-CCl_4), and CCl_4 plus butanol extract of KF (KFB-CCl_4) groups. KFM and KFB were orally administered once a day (200 mg/kg body weight) for 14 days. A mixture of 0.2 mL/100 g body weight of CCl_4 in olive oil was injected at 30 minutes after the final administration of KFM and KFB. The KFB pretreatment resulted in a significant decrease in the serum transaminase and lactic dehydrogenase levels in the CCl_4-treated rats. The CCl_4 treatment significantly lowered the activities of glutathione, glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px). However, pretreatment with KFM and KFB resulted in a significant increase in the glutathione, GR and GST levels. KFB increased the activities of SOD, catalase and GSH-Px, but KFM did not alter them. Pretreatment with KFM and KFB resulted in a significant decrease in the production of aminopyrine N-demethylase in the CCl_4-treated rats. KF extract would appear to contribute to alleviate the adverse effect of CCl_4 treatment by enhancing the hepatic antioxidant defense system.

Key words: *Kochiae fructus*, Carbon tetrachloride, Hepatotoxicity

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INTRODUCTION

The liver has been called the metabolic capital of the body. Through its vast network of biochemical reactions, the liver controls a major portion of the body's internal environment. Hepatic injury is associated with distortion of these metabolic functions. Liver disorder remains one of the serious health problems.[1)] Despite of tremendous strides in the modern medicine, there are not many drugs available for the treatment of liver disorders.[2)]

Medicinal plants are currently being investigated for their pharmacological properties. *Kochia scoparia*, an annual herbaceous plant reaching 50 to 150 cm in height, is cultivated in Far East Asia. The fruit of this plant (*Kochiae fructus* KF, Jibuja in Korean) is known as one of traditional oriental medicine.[3)] KF has been used as a tonic, diuretic, analgesic, and antidote and for the treatment of cutaneous pruritus in Korean traditional preparations. It has been mentioned for the treatment of thermal skin diseases and liver disorders and used in traditional medicine due to its alleviation effect of jaundice and edema.[4,5)] But this plant has not been subjected to systematic investigation to assess its hepatoprotective effect.

Carbon tetrachloride (CCl_4) is widely used to treat animals in the liver injury model, because the damage by CCl_4 is regarded as the analogue of liver damage caused by a variety of hepatotoxins in humans. CCl_4-induced liver damage has been thought to depend on the formation of reactive intermediates such as trichloromethyl free radical produced by cytochrome P450 monooxygenase system,[6)] and further converted to a peroxy radical.[7)] Accordingly, the current study evaluated the hepatoprotective effect of KF extract against CCl_4-induced liver damage in rats based on analyzing antioxidant enzymes, microsomal aniline hydroxylase, aminopyrine N-demethylase, and serum transaminase.

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MATERIALS AND METHODS

1. Preparation of Plant Extract

Kochiae fructus (KF) was collected from Gangweon-do Province of Korea and identified by Dr. H. J. Park, Professor, Department of Botanical Resources, Sangji University (voucher specimen No. SJHJ136). The dried KF (1 kg) was powdered and extracted 3 times with methanol (3 liters) at 60 ℃ for 3 hours. After filtration, the methanol solution was concentrated with vacuum rotary evaporator and freeze-dried. The methanol extract (KFM) was dissolved in distilled water and extracted with chloroform. The aqueous layer was concentrated under reduced pressure and freeze-dried. Then the extract was dissolved in distilled water and partitioned with ethyl acetate. The aqueous layer was concentrated under reduced pressure and freeze-dried. Also, the extract was dissolved in distilled water and extracted with butanol. The butanol extract (KFB) was concentrated and freeze-ried.

2. Animals

Thirty two male Sprague-Dawley rats weighing between 110 and 120 g were purchased from Daehan Biolink Co. (Daegu, Korea). The animals were all individually housed in stainless steel cages in an air-conditioned room with controlled temperature (20 ~ 22 ℃) and automatic lighting (alternation 12-h periods of light and dark) and fed an AIN-93[8] standard laboratory diet for 14 days after arrival. During thepreliminary investigation, the chloroform extract of KF and the ethyl acetate extract of KF did not produce any significant effect in the transaminase level and antioxidant enzyme activities. So the animals were randomly divided into four groups (n=8) which were assigned to one of four dietary categories, a control, CCl_4 treated, CCl_4 plus KFB treated (KFB-CCl_4) group and CCl_4 plus KFM treated (KFM-CCl_4) group. KFB (200 mg/kg body weight) and KFM (200 mg/kg body weight) were orally administered once a day for 14 days. A mixture of 0.2 mL/100 g body weight of CCl_4 in olive oil (1:1 v/v) was injected at 30 minutes after the final administration of KFB and KFM. The control group was given saline solution.

The rats were given free access to food and distilled water. The food consumption and body weight gain were measured daily and weekly, respectively. At the end of the experimental period, the rats were anesthetized with ether following a 12-h fast. Blood samples were taken from the abdominal aorta to determine serum aspartate transaminase (AST), serum alanine transaminase (ALT) and lactic dehydrogenase (LDH). The livers were removed and rinsed with physiological saline. All samples were stored at-70 ℃ until analyzed. The approval of this experiment was obtained from the Animal Care and Use Committee of the Kosin University.

3. Tissue Preparations

The livers were homogenized in 20 parts (w/v) of 0.25 M sucrose solution using a tissue homogenizer with a Teflon pestle at 4 ℃. The homogenate was centrifuged at 600 × g for 10 min to discard any cell debris, and then the supernatant was further centrifuged at 10,000 × g for 20 min to remove the mitochondria pellet. The supernatant was further ultracentrifuged at 105,000 × g for 60 min to obtain the cytosol supernatant. The pellet was suspended in potassium phosphate buffer (0.1 M, pH 7.4) and ultracentrifuged at 105,000 × g for 60 min to obtain the microsomal fraction. The amounts of protein in the mitochondrial, cytosolic and microsomal fractions were measured using the method of Lowry *et al.*[9] with bovine serum albumin as the standard.

4. Serum Aminotransferase and Lactic Dehydrogenase Activities

The serum AST and ALT activities were determined using a commercial kit (Eiken Co., Tokyo, Japan) based on the method of Reitman and Frankel.[10] Serum LDL was determined using a commercial kit (Asan Chemical Co., Seoul, Korea) based on the method of Berga and Boida.[11]

5. Antioxidant Enzyme Activities and Thiobarbituric Acid-reactive Substances (TBARS) Concentration

The level of hepatic malondialdehyde (MDA) was measured using the method of Ohkawa *et al.*[12] Two hundred microliters of 20% (w/v) liver homogenate was mixed with 600 μl of distilled water and 200 μl of 8.1% (w/v) sodium dodecyl sulfate (SDS), vortexed, and incubated at room temperature for 5 min. After adding 1.5 mL of 20% (w/v) acetic acid (pH 3.5) and 1.5 mL of 0.8% (w/v) thiobarbituric acid (TBA), the mixture was heated at 95 ℃ for 60 min. After cooling with tap water, 1 mL of distilled water and 5.0 mL of a mixture of n-butanol and pyridine (15:1, v/v) were added and vortexed. Then after centrifugation at 800 × g for 15 min, the absorbance of the upper layer was measured at 532 nm using 1,1,3,3-tetraethoxypropane (Sigma chemical Co., St. Louis, MO) as the standard.

The superoxide dismutase (SOD) activity was measured using the method of Marklund and Marklund.[13] Twenty

microliters (350 ~ 400 μg protein) of the cytosol supernatant was mixed with 2.88 mL of 50 mM Tris-HCl buffer (pH 8.2) with 10 mM EDTA, then 100 μl of 15 mM pyrogallol was added and the incubation mixture was measured at 440 nm for 10 min on a spectrophotometer. One unit of activity was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as units/mg protein.

The catalase (CAT) activity was measured using the method of Abei.[14] The mitochondria pellet was dissolved in 1.0 mL of 0.1 M potassium phosphate buffer (pH 7.4). Ten microliters (120 ~ 150 μg protein) of the mitochondria solution was then added to a cuvette containing 2.89 mL of 50 mM phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1 mL of freshly prepared 30 mM H_2O_2 to make a final volume of 3.0 mL at 25 ℃. The decomposition rate of H_2O_2 was measured at 240 nm for 5 min on a spectrophotometer. A molar extinction coefficient of 0.04 l/mM/cm was used to determine the CAT activity, which was then expressed as nM H_2O_2 decreased/mg protein/min.

The glutathione peroxidase (GSH-Px) activity was measured using the method of Paglia and Valentine.[15] The reaction mixture contained 2.525 mL of 0.1 M Tris-HCl buffer (pH 7.2), 75 μl of 0.04 M GSH (reduced form), 100 μl of 0.1 M nicotinamide adenine dinucleotide phosphate (NADPH), and 100 μl of glutathione reductase (0.24 unit). One hundred microliters (1.75 ~ 2.00 mg protein) of the cytosol supernatant was added to 2.8 mL of the reaction mixture and incubated at 25 ℃ for 5 min. The reaction was initiated by adding 100 μl of 0.75 mM H_2O_2, and then its absorbance was measured at 340 nm for 5 min. The activity was expressed as nmol NADPH oxidized/mg protein/min using a molar extinction coefficient of 6.22 × 10^3/mM/cm.

6. Glutathione Measurement

The glutathione (GSH) content was measured using the method of Ellman.[16] Five hundred microliters of the liver homogenate was mixed with 500 μl of 4% (w/v) sulfosalicylic acid and centrifuged at 600 × g for 10 min. Three hundred microliters of the supernatant was added to 2.7 mL of disulfide (5,5'-dithiobis-2-nitrobenzoic acid) reagent, and measured at 412 nm. Total GSH content was expressed as nmole/g of tissue.

7. Glutathione-S-Transferase and Glutathione Reductase Activities

The glutathione-S-transferase (GST) activity was estimated by the method of Habig *et al.*[17] The reaction mixture consisted of 2.5 mL sodium phosphate buffer (0.1 M, pH 7.4), 0.1 mL reduced glutathione (1 mM), 0.1 mL PMS (10%, w/v) in a total volume of 3.0 mL. The changes in the absorbance were recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar coefficient of 9.6 × 10^3/M/cm.

The glutathione reductase (GR) activity was assayed by the method of Carlberg and Mannervik[18] and then modified according to Mohandas *et al.*[19]

8. Aniline Hydroxylase and Aminopyrine N-demethylase Activities

The aniline hydroxylase (AH) was assayed by the method of Rajagopalan *et al.*[20] by measuring ρ-aminophenol formed from aniline hydrochloride at 37 ℃ (pH 7.4). The aminopyrine N-demethylase (AD) was assayed by the Nash's method[21] by estimating formaldehyde formed during N-demethylation of aminopyrine at 37 ℃ (pH 7.5).

9. Statistical Analysis

All data are presented as the Mean±SE. The data were evaluated by a one-way ANOVA using the SPSS program, and the differences between the means were assessed using Duncan's multiple range test. Statistical significance was considered at $p<0.05$.

RESULTS

1. Serum Aminotransferase and LDH Activities

The CCl_4-treated groups resulted in a significant increase in serum AST, ALT and LDH levels compared to the control group (Table 1); ALT and LDH levels were lower in the KFB-CCl_4 group than in the CCl_4 group. The AST levels were significantly lower in the KF extract supplemented groups than in the CCl_4 group.

Table 1. Effect of Kochiae Fructus on serum aspartate transaminase (AST), alanine transaminase (ALT) and lactic dehydrogenase (LDH) in CCl_4-intoxicated rats

Groups	AST	ALT	LDH
Control	37.5± 8.7^d	26.5±5.7^c	41.7± 9.4^c
CCl_4	194.5±10.6^a	110.4±7.8^a	180.6±10.8^a
KFM-CCl_4	106.4± 8.4^b	107.5±9.4^a	164.4± 9.6^a
KFB-CCl_4	145.7± 9.8^c	73.7±4.0^b	124.8± 8.7^b

Units: AST, unit/mL; ALT, unit/mL; LDH, nmol NADH oxid/mg protein/min.
Values are Mean±SE of 8 rats from each group.
^{abc} Means in the same column not sharing a common superscript are significantly different ($p<0.05$) between groups.

2. Effect on Hepatic Lipid Peroxidation

The concentration of hepatic TBARS is shown in Fig. 1. The TBARS level in the liver of rats on CCl_4 treatment was significantly higher as compared with those of the control rats. Pretreatment with KFM and KFB to rats on CCl_4 treatment lowered the TBARS levels. The content of GSH in the CCl_4-treated rats was significantly decreased (Fig. 2), and the pretreatment with 200 mg/kg of KFM and KFB increased it significantly.

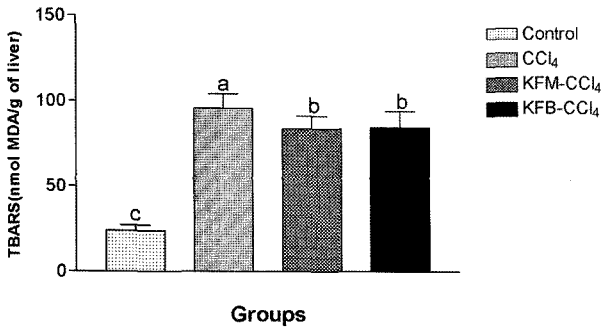

Fig. 1 Hepatic thiobarbituric acid reaction substances (TBARS) level (Mean±SE) in experimental groups.
The TBARS values are expressed as nmole MDA/g of liver.
The means sharing a common letter are not significantly different ($p<0.05$) between groups.

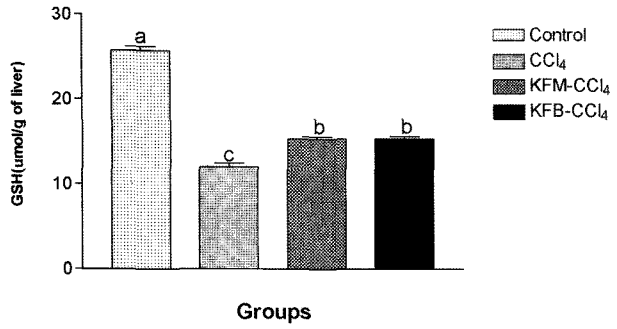

Fig. 2 Glutathione level (Mean±SE) in experimental groups.
The GSH values are expressed as μmole/g of liver.
The means sharing a common letter are not significantly different ($p<0.05$) between groups

The activities of SOD, CAT and GSH-Px of control and experimental animals are given in Table 2. SOD, CAT and GSH-Px activities in the liver of the CCl_4-treated rats were significantly lower than those of the control rats. Pretreatment with KFM to the CCl4treated rats did not alter the SOD, CAT and GSH-Px activities significantly as compared to the CCl_4 group. Whereas pre-treating KFB to CCl4-treated rats significantly elevated their activities as compared with those on CCl_4 treatment alone.

Table 2. Effect of Kochiae Fructus on Antioxidant Enzyme Activities in CCl_4-treated rats

Groups	SOD	Catalase	GSH-Px
Control	5.55±0.64[a]	36.97±0.90[a]	7.98±0.74[a]
CCl_4	2.36±0.47[c]	13.34±0.67[c]	4.59±0.92[c]
KFM-CCl_4	2.95±0.44[c]	15.09±2.57[c]	4.49±0.21[c]
KFB-CCl_4	3.76±0.65[b]	23.33±0.66[b]	5.09±0.87[b]

Abbreviation used and units: SOD, superoxide dismutase as unit/mg protein; GSH-Px, glutathione peroxidase as decreased NADPH nmole/mg protein/min; catalase as decreased H_2O_2 nmole/mg protein/min.
Values are mean±SE of 8 rats from each group.
[ab] Means in the same column not sharing a common superscript are significantly different ($p<0.05$) between group.

3. Effect on GR and GST Activities

The activities of GR and GST are shown in Table 3. The GR and GST activities of the CCl_4-treated rats were significantly lower than those of the control rats. The pretreatment with KFM and KFB to CCl_4-treated rats increased the reduced levels of GST and GR.

Table 3. Effect of Kochiae Fructus on hepatic glutathione reductase (GR) and glutathione-S-transferase (GST) in CCl_4-intoxicated rats

Groups	GR	GST
Control	28.91±3.16[c]	253.7±19.3[a]
CCl_4	11.62±2.10[d]	122.6± 9.4[d]
KFM-CCl_4	15.40±1.56[c]	140.5±10.7[c]
KFB-CCl_4	18.51±1.78[b]	157.7±15.4[b]

Units: GR, GSH nmol/mg protein/min; GST, nmol/mg protein/min.
Values are meanSE of 8 rats from each group.
[abcd] Means in the same column not sharing a common superscript are significantly different ($p<0.05$) between groups.

4. Effect on AD and AH

The activities of AD and AH are shown in Table 4. The AD and AH activities in the liver of the CCl_4 treated rats were significantly higher than those of the control rats. Pretreatment with 200 mg/kg of KFM and KFB reduced CCl_4-induced AD production. Pretreatment with 200 mg/kg of KFB reduced CCl_4-induced AH production, but pretreatment with 200 mg/kg of KFM to the CCl_4-

Table 4. Effect of Kochiae Fructus on serum aminopyrine N-demethylase (AD) and aniline hydroxylase (AH) in CCl_4-intoxicated rats

Groups	AD	AH
Control	5.65±0.61[c]	2.07±0.36[c]
CCl_4	20.04±0.56[a]	11.77±3.33[a]
KFM-CCl_4	11.05±0.93[b]	10.42±1.84[a]
KFB-CCl_4	11.08±0.79[b]	7.45±0.99[b]

Unit: AD, HCHO nmol/mg protein/min; AH, p-aminophenl nmol/mg protein/min.
Values are mean±SE of 8 rats from each group.
[abc] Means in the same column not sharing a common superscript are significantly different ($p<0.05$) between groups.

treated rats did not alter the AH activity significantly as compared to the CCl_4-treated group.

DISCUSSION

Carbon tetrachloride induces hepatotoxicity by metabolic activation, and therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. Carbon tetrachloride is metabolically activated by the cytochrome P450-dependent mixed oxidase in the endoplasmic reticulum to form trichloromethyl free radical which is combined with cellular lipids and proteins in the presence of oxygen to induce lipid peroxidation.[22,23] These result in changes of structures of the endoplasmic reticulum and other membranes, loss of metabolic enzyme activation, reduction of protein synthesis and loss of glucose-6-phosphatase activation, leading to liver damage.[24-26]

The damage to cell membrane integrity causes the release of cellular hepatospecific enzymes, mainly the transaminases (AST, ALT). After CCl_4 treatment, the AST, ALT and LDH levels in serum increased significantly, as expected. The abnormally higher levels of serum ALT, AST, and LDH in our study are the consequences of carbon tetrachloride induced liver dysfunction and denote the damage to the hepatic cells.[27] The activities of AST and ALT are the most sensitive tests employed in the diagnosis of hepatic diseases.[28] In agreement with the present study, it has been found that treatment of mice with CCl_4 induced the activity of LDH which can be used as a marker for hepatotoxicity.[29]

In the present study, pretreatment with KFB attenuated the increases in the activities of AST, ALT, and LDH produced by CCl_4, indicating that KFB protects liver injury induced by CCl_4. KFM pretreatment did not produce any significant effect in ALT and LDH activities. KFB is more effective than KFM in recovering the alteration in ALT and LDH activities in the CCl_4 rats, although the liver was not quite recovered to the normal level.

Glutathione (GSH) is an important cellular factor influencing the effectiveness of a variety of chemotherapeutic and alkylating agents, and organs with low GSH levels are more susceptible to the action of these agents.[30-32] Glutathione constitutes the first line of defense against free radicals. Reduction in liver GSH and decrease in GSH-Px activity in CCl_4-treatedted rats as observed in our study indicated the damage to the hepatic cells, which are consistent with earlier reports.[27]

Glutathione reductase maintains GSH in a reduced form whereas GSH-Px utilizes it for the decomposition of lipid hydroperoxides and other reactive oxygen species.[33] Substantial depletion of hepatic GSH and a concomitant decrease in the activities of GR and GSH-Px, on CCl_4 administration, were significantly alleviated by the pretreatment of rats with KFM and KFB. Therefore it is assumed that effects of KFB and KFM on liver protection are related to the glutathione-mediated detoxification as well as free radical suppressing activity

Lipid peroxidation chain reaction generates a principal product called malondialdehyde (MDA). Glutathione is the strong nucleophilic molecule found in most cells with its function as an antioxidant. Free radicals are usually destroyed by GSH. Carbon tetrachloride induced cell death may be determined by increasing the release of cellular transaminases, increasing the production of MDA and decreasing cellular level of GSH.[6] It has been hypothesized that KFM and KFM can afford the protection by impairing CCl_4 mediated lipid peroxidation, through the decreased production of free radical derivatives, as evident from the ameliorated TBARS levels. This result indicates that the inhibition of lipid peroxidation may be one mechanism through which KFM and KFB extract can protect against the liver damage mediated by CCl_4. The fact that the plant extract can maintain the level of GSH in the liver suggests that it acts by preventing irreversible binding of CCl_4 to some important cellular proteins.[34]

Glutathione-S-transferase has been considered as an early marker of CCl_4 hepatotoxicity in rats. It catalyzes the conjugation of the thiol functional groups of glutathione to electrophilic xenobiotics and results in increasing solubility. The xenobiotic-GSH conjugate is then either eliminated or converted to mercapturic acid.[35,36] Pretreatments with KFM and KFB in the CCl_4-treated rats were beneficial in increasing the GST activity. It would seem that KFM and KFB pretreatments could be used to improve the protective activity of the liver.

The hepatotoxicity of CCl_4 is thought as the result from its reductive dehalogenation by cytochrome P-450 into the trichloromethyl free radical.[37] These radicals and the corresponding peroxyl radicals create lipid radicals, and thereby initiatea process of lipid peroxidation. Enhanced lipid peroxidation (expressed in terms of TBARS) and reduced activities of SOD and CAT observed in CCl_4-treated rats in our study (Table 2) confirm the hepatic damage to the rats.[38] Pretreatments of KFM and KFB to the rats in which hepatic damage was induced by CCl_4 treatment, caused significant decrease in lipid peroxidation. This indicates the anti-lipid peroxidative

nature of the system as brought about by the KFM and KFB against the damaging effects of free radicals produced by CCl_4.

Activities of SOD and CAT, which can help to scavenge superoxide ions and hydroxyl ions respectively, were significantly lower in CCl_4-treated rats compared with those of control rats. Catalase has been reported to be responsible for the detoxification of H_2O_2, which is an effective inhibitor of SOD.[39] The decrease in the CAT activity could be due to either the depletion or inhibition of the enzyme as a result of the increased production of free radicals.[40] The decrease in the SOD activity could be due to the oxidative inactivation of the enzyme as a result of excessive production of reactive oxygen species[41] or the generation of -hydroxy ethyl radicals that inactivate the SOD enzyme.[42] Pretreatment with KFB to the CCl_4-treated rats significantly elevated SOD and CAT activities compared tothose on the CCl_4 treatment alone. Pretreatment with KFM had no significant effect on SOD and CAT activities. An increase in the SOD activity may protect CAT against enzyme inactivation by O^2-anions as these anions have been shown to inactive CAT.[40] A KFB supplement can partially reduce the imbalance between the generation of reactive oxygen species and the scavenging enzyme activity.

Living organismsare exposed to continual influence of foreign organic compounds of heterotrophic origin. The main response of an organism is to detoxify the xenobiotics to biologically inactive and readily excretable compounds. This route of metabolism employs a wide range of different enzymes and is normally divided into two phases, known as phase I or functional reactions and phase II or conjugative reactions. Of great importancein phase I metabolism are oxidative reactions catalyzed by the cytochrome P450-dependent monooxygenase system. The activity of cytochrome P450-dependent system is inducible by exposure to organic xenobiotics, which are subsequently metabolized.[43] It is known that organochlorines following microsomal metabolism by the P-450 oxidase system undergo covalent binding to hepatic microsomal protein.[44] Our data (Table 4) show that pretreatment with KFB and KFM to CCl_4-treated rats preserved the phase I drug metabolizing enzymes.

KF extract would appear to contribute to alleviate the adverse effect of CCl_4 treatment by enhancing the hepatic antioxidant defense system. However, although detoxification action of KF extract was clearly beneficial for CCl_4-treated rats, the detoxification mechanism at the pharmacological and biochemical level still needs to be elucidated.

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