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Protective Effects of Hot Water Extracts from Safflower (*Carthamus tinctorius* L.) Petals on Paraquat-Induced Oxidative Stress in Rats

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The protective effects of hot water extracts from safflower petals (Ex) against paraquat (PQ)-induced oxidative were examined in rats. Ex significantly relieved decreases in food intake and body weight gain, and increase in lung weight which were induced by the administration of the paraquat diet. Ex also relieved increases in liver TBARS, and decreases both in the catalase activity in liver mitochondrial fraction and the level of liver triacylglycerol in the PQ-fed rats. These results indicate the possibility that Ex acts preventively against oxidative stress.

Keywords: safflower, oxidative stress, paraquat, antioxidative enzyme activity

The safflower (*Carthamus tinctorius* L.) has been well known for centuries in China and Japan and has often been used as a medicinal herb to cure menstrual irregularity, oversensitivity to cold, and menopausal and blood circulation disorders (Mizuno & Yoneda, 1983). The antitumor and antiallergic activities of the safflower have been studied (Toyoda *et al.*, 1997) and more recently their physiological functions have been extensively investigated (Kawashima *et al.*, 1998).

Safflower is also drunk as tea in Japan, making it of great interest to determine the physiological functions of the hot water extract. In this study, we examined whether water extracts of safflower petals can prevent oxidative stress in rats which was induced by paraquat (PQ), a reagent to produce active oxygen species through redox cycling by microsomal NADPH-cytochrome P-450 reductase (Bismuth *et al.*, 1990).

Material and Methods

Preparation of extract Safflower was obtained from a farmhouse in Tsuruoka-shi in Yamagata in Japan. The petals removed from the fresh safflower within 6-h after harvest were stored at −20°C until extraction with hot water. The frozen safflower petals were placed in boiling water for 30 min and the filtrate of the extract was subsequently freeze-dried (Ex). The extract yield was about 10 g from 100 g of fresh petal. Ascorbic acid, which is known to have radical scavenging activity, was not detected in the Ex.

Animal and diets Male rats of the Wistar-strain were purchased when they were four-weeks-old and about 50 g in body weight, from Japan SLC (Hamamatsu). They were given a commercial pellet diet and water for 2 days before use in the experiment.

The basal diet (C) contained (by weight) 20% casein, 5% corn oil, 5% cellulose, 3.5% mineral mixture (AIN-93G-MX), 1% vitamin mixture (AIN-93-VX (Reeves *et al.*, 1993), Oriental Yeast Co., Tokyo), and 65.5% of a mixture of α-cornstarch and sucrose with a mixing ratio of 2 to 1. For the Ex-added diet (+Ex), Ex was added to the basal diet at a ratio of 0.83% instead of the mixture of α-cornstarch and sucrose. The paraquat diet (+PQ diet) and paraquat plus Ex diet (+PQ+Ex diet) were made by adding 0.02% paraquat and 0.02% paraquat and 0.83% Ex, respectively. The addition level of 0.83% is equal to the 0.05% level of safflomin a, which is a major yellow component in safflower petals (Onodera *et al.*, 1981), in the basal diet.

Collection and preparation of blood samples and liver samples Blood was collected via a cardiac puncture from rats anesthetized with Nembutal (50 mg/ml; Dainippon Pharmaceutical Co., Osaka) after 10 h of starvation at the end of the feeding period. One-tenth milliliter aliquot of the blood was added to 1.9 ml of physiological saline by gently shaking, and the mixture was centrifuged at 1000×*g* for 10 min to obtain the serum for measuring the serum TBARS concentration. A section of the right lobe of the liver which had been excised from each of the rats was used to measure the liver superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase, and glutathione reductase (GSSG-R) activities as well as the liver thiobarbituric acid-reactive substances (TBARS). The other parts of the liver were stored at −30°C until needed to measure the concentrations of lipids. Centrifuging the blood at 1000×*g* for 15 min separated the serum so that lipids could be measured.

Preparation of subcellular fraction A homogenate for measuring liver TBARS and a supernatant for measuring antioxidative enzyme activities in the liver mitochondrial and cytosolic fractions were prepared according the method of Del Boccio *et al.* (1990), with slight modifications as previously reported (Igarashi *et al.*, 1998).

SOD and catalase activities were measured by the methods of Imanari *et al.*(1977), and of Tomita and Sano (1983),

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respectively. The GSH-Px activity was measured using *t*-butyl hydroperoxide as a substrate by the methods of Lawrence and Burk (1976), and of He and Yasumoto (1990). GSSG-R activity was measured by the method of He and Yasumoto (1990). The definition of one unit of SOD and catalase activity is the amount of enzyme required to inhibit the rate of diformazan formation from NBT by 50% per mg of hemoglobin or protein, and the amount of enzyme needed to decompose 1 μmol of H_2O_2 per min per mg of hemoglobin or protein, respectively. The definition of one unit of GSH-Px and GSSG-R activity is the amount of enzyme required to oxidize 1 μmol of NADPH per min per mg of hemoglobin or protein.

The hemoglobin and protein contents were measured using a commercial hemoglobin test kit (Wako Pure Chemical Ind., Osaka) and by the method of Lowry *et al.*(1951),respectively.

Measurement of TBARS The serum TBARS concentration was determined by the method of Yagi (1976) and is expressed as nmol of malondialdehyde per ml of blood. To determine the TBARS concentrations in liver and lungs, fresh liver and frozen lungs were homogenized with ice-cold 0.1 M phosphate buffer (ph 7.4) containing 1 mM EDTA, respectively and the homogenate was then mixed with 2 volumes of 2.3% KCl (Del Boccio *et al.*, 1990). TBARS of the homogenate was measured by the method of Uchiyama and Mihara (1978).

Lipid analyses The total cholesterol, triacylglycerol, and phospholipid in the serum were enzymatically measured using commercial kits (cholesterol E-test, triglyceride E-test, and phospholipid B-test, respectively; Wako Pure Chemical Ind.). Serum HDL-cholesterol was enzymatically measured in the supernatant obtained after the heparin-Mn precipitation of the other lipoproteins (Burstein *et al.*, 1970), using a commercial kit (HDL-cholesterol, Wako Pure Chemical Ind.)

The lipids from a frozen liver were extracted and purified by the method of Folch *et al.* (1957). The lipids in the extract were measured using the same kits as those for the serum except that a Mono-test cholesterol (Boehringer Mannheim Yamanouchi Co., Tokyo) was used instead of the cholesterol E-test.

Preparation of lung samples The lungs excised from each rat immediately after the collection of blood and liver were stored at −30°C until the TBARS and antioxidative enzyme activities could be determined. An enzyme solution to determine SOD, catalase and GSH-Px activities was prepared from the frozen lungs according to the method of Paynter *et al.* (1979).

Statistical analyses Data for each of the 4 groups were statistically analyzed by Duncan's multiple range test after a two-way analysis of variance (ANOVA) with the exception that food intake and body weight after nine days were compared using the Mann-Whitney test for their nonparametric data. Significant differences in means were analyzed at $p<0.05$.

Results and Discussion

Food intake and body and lung weight Food intake and body weight tended to decrease after 9 days in rats fed a paraquat diet (+PQ group), compared with the figures in the control rats. The supplements of Ex in the paraquat diet relieved decreases in both food intake and body weight. An increase in the lung weight in the paraquat-fed rats was relieved by supplementing Ex to the paraquat diet, and the interaction between paraqaut and Ex was noted in the lung weight (Table 1). These results suggest that oxidative stress by paraquat may be relieved by ingesting Ex; lung weight is known to increases with administration of paraquat to rats (Abe, 1987).

TBARS and antioxidative enzyme activities As shown in Table 1, although the serum TBARS concentration did not differ among the 4 groups, the liver TBARS concentration in the paraquat-fed rats tended to increase compared with that of the control rats. The addition of Ex to the paraquat diet tended to limit this increase. However, when the liver TBARS concentration was compared on the basis of nmol TBARS per mg liver lipid, the addition of Ex to the paraquat diet significantly suppressed the increase in TBARS due to paraquat, and an interaction between paraquat and Ex was observed. These results suggest that Ex can effectively relieve an increase in TBARS due to administration of the paraquat.

As shown in Table 1, the catalase activity in the liver mitochondrial fraction decreased markedly in paraquat-fed rats, and tended to return to the level of the control rats by supplementing Ex to the paraquat diet. A significant interaction between paraquat and Ex in the catalase activity of the mitochondrial fraction indicates that Ex can suppress the decrease in catalase activity that is caused by paraquat. The GSSG-R activity in the liver cytosol fraction was higher in rats fed the paraquat diet supplemented with Ex than in the paraquat-fed rats, and an increasing effect of Ex on the GSSG-R activity was observed.

Lipid levels As shown in Table 2, the serum total cholesterol, HDL-cholesterol, triacylglycerol, and phospholipid concentrations were not statistically different between the control and Ex-fed rats, or between the paraquat-fed rats and those fed a paraquat diet supplemented with Ex. However, the liver triacylglycerol concentration markedly decreased when the rats were fed the paraquat diet. The values that were decreased tended to return to the level of the control rats when Ex was supplemented in the paraquat diet, but not to a level equal to that of the control rats. This may be partly due to the liver triacylglycerol-lowering effects of Ex, because this level tended to decrease in Ex-fed rats compared with that of the control rats.

Lung TBARS and antioxidative enzyme activities As shown in Table 1, the level of lung TBARS and the lung SOD and GSH-Px activities did not differ among the 4 groups to a statistically significant level; however, the lung catalase activity which tended to decrease by administration of the paraquat diet showed a significant increase with the use of the paraquat-added diet which was supplemented with Ex, and showed an interaction between paraquat and Ex. These results suggest that Ex can increase catalase activity in the presence of paraquat, and can protect lungs from oxidative stress by paraquat.

In this experiment, the addition level of Ex to the basal diet

Table 1. Effects of hot water extracts of safflower petals on organ weight, TBARS levels in serum, liver and lung, and on the liver and lung antioxidative enzyme activities of rats fed on diets with or without paraquat.

	Diet group				ANOVA (P value)		
	C[1]	+PQ	+Ex	+PQ+Ex	PQ[1]	Ex[2]	PQ×Ex[3]
Initial body weight(g)	58.7±1.9	58.8±1.9	58.7±1.6	58.8±2.0	NS	NS	NS
Final body weight (g)	98.2±1.0^{a}	70.8±4.4^{b}	99.4±2.0^{a}	93.6±2.6^{a}	<0.002	<0.002	<0.002
Liver weight (g/100 g of body weight)	4.08±0.2	3.89±0.06	3.96±0.14	3.66±0.11	NS	NS	NS
Lung weight (g/100 g of body weight)	0.58±0.01^{c}	1.18±0.79^{a}	0.55±0.01^{c}	0.68±0.01^{b}	<0.002	<0.002	<0.002
Serum TBARS (nmol/ml of blood)	1.87±0.13	1.80±0.21	1.73±0.13	1.64±0.16	NS	NS	NS
Liver TBARS (nmol/g of liver)	39.5±1.4	47.3±3.4	39.4±1.3	37.3±3.0	NS	NS	NS
Liver TBARS (nmol/mg of lipid)	0.85±0.04^{b}	1.32±0.10^{a}	0.95±0.04^{b}	0.92±0.07^{b}	<0.006	NS	<0.002
Lung TBARS (nmol/g of lung)	131±7^{ab}	120±5^{b}	138±1^{a}	126±8^{ab}	NS	NS	NS
Antioxidative enzyme activities							
in liver							
in cytosol fraction							
SOD (U/mg of protein)	3.14±0.16	3.01±0.12	3.04±0.08	3.11±0.09	NS	NS	NS
Catalase(U/mg of protein)	265±18^{ab}	262±3^{a}	243±8^{ab}	228±9^{b}	NS	<0.021	NS
GSH-Px (U/mg of protein)	0.70±0.06	0.58±0.05	0.68±0.02	0.62±0.05	NS	NS	NS
GSSG-R (U×10^{-2}/mg of protein)	9.81±0.42^{b}	10.1±0.4^{b}	10.4±0.2^{b}	12.0±0.7^{a}	NS	<0.016	NS
in mitochondria fraction							
SOD (U/mg of protein)	4.48±0.52	2.92±0.28	3.72±0.37	3.54±0.40	<0.047	NS	NS
Catalase (U/mg of protein)	281±38^{a}	88±7^{b}	226±23^{a}	176±36^{a}	<0.002	NS	<0.025
in lung							
SOD (U/mg of protein)	2.99±0.08	3.45±0.23	3.02±0.10	2.84±0.17	NS	NS	NS
Catalase (U/mg of protein)	33.8±1.5^{bc}	27.5±1.6^{c}	35.8±2.1^{b}	42.8±3.4^{a}	NS	<0.002	<0.010
GSH-Px (U×10^{-2}/mg of protein)	6.7±1.2	6.3±0.6	4.6±1.0	5.8±1.0	NS	NS	NS

Values are mean±SE of 5 or 6 rats per group. [1]Effects of paraquat (PQ) in diets. [2]Effects of hot water extracts of safflower petals (Ex) in diets. [3]Interaction between PQ and Ex. Within rows, means with different superscript letters are significantly different ($p<0.05$).

Table 2. Effects of hot water extracts of safflower petals on the serum and liver lipid concentrations of rats fed diets with or without paraquat.

	Diet group				ANOVA (P value)		
	C	+PQ	+Ex	+PQ+Ex	PQ[1]	Ex[2]	PQ×Ex[3]
Serum lipids							
Total cholesterol (mmol/l)	1.86±0.06^{a}	1.96±0.23^{ab}	1.99±0.08^{a}	1.65±0.04^{b}	NS	NS	NS
HDL-cholesterol (mmol/l)	1.47±0.03	1.58±0.18	1.64±0.09	1.42±0.07	NS	NS	NS
Triacylglycerol (mmol/l)	0.55±0.12^{a}	0.23±0.03^{b}	0.54±0.11^{a}	0.36±0.07^{ab}	< 0.012	NS	NS
Phospholipid (mmol/l)	1.45±0.10	1.19±0.17	1.52±0.05	1.16±0.10	< 0.012	NS	NS
Liver lipids							
Total cholesterol (μmol/g of liver)	4.74±0.19	4.58±0.15	4.63±0.15	4.92±0.18	NS	NS	NS
Triacylglycerol (μmol/g of liver)	11.6±1.7^{a}	1.94±0.49^{c}	8.03±0.91^{ab}	5.49±0.83^{b}	<0.002	NS	<0.004
Phospholipid (μmol/g of liver)	16.7±0.2	17.4±0.2	16.8±0.3	17.9±0.6	< 0.025	NS	NS

Values are mean±SE of 5 or 6 rats per group. [1]Effects of paraquat (PQ) in diets. [2]Effects of hot water extracts of safflower petals (Ex) in diets. [3]Interaction between PQ and Ex. Within rows, means with different superscript letters are significantly different ($p<0.05$).

used was 0.83% which corresponded to 0.05% safflomin a, a major yellow pigment in safflower petals, in the basal diet. Although safflomin a showed weak radical scavenging activities for hydroxy radical and superoxide anion when determined with ESR equipment, its addition to the paraquat diet at 0.05% did not prevent oxidative stress induced by paraquat to a statistically significant degree (data not shown). This result indicates that compounds other than safflomin a may contribute to protection against paraquat-induced oxidative stress. Although flavonoids which scavenged DPPH (1,1-diphenyl-2-picrylhydrazyl) radical were detected as minor components along with major components such as sugars and free amino acids in the Ex, the components which are effective in suppressing oxidative stress remain to be identified. As the amount of Ex administered to a rat (about 100 g body weight) per day corresponded to 1 g of fresh safflower petals, 600 g of fresh petals may be necessary to obtain the same effect in a person (60 kg body weight). However, the drinking of Ex over a long period may allow a decrease in the daily amount necessary to protect against oxidative stress.

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