Anti-lipid Peroxidative Effect of an Extract of the Stems of *Kadsura heteroclita* and Its Major Constituent, Kadsurin, in Mice

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Three-days successive *p.o.* administration of an EtOH extract of the stems of *Kadsura heteroclita* (Schizandraceae) or its major constituent, kadsurin, resulted in significant decreases of CCl_4-induced lipid-peroxidation products, such as thiobarbituric acid reactive substances (TBA-RS), conjugated dienes and fluorescent products in the liver of mice. In contrast, a significant restoration of superoxide dismutase (SOD) activity reduced by CCl_4-intoxication was observed in the administered groups, suggesting that the subchronic treatment of mice with the EtOH extract or kadsurin induce enzymes capable of scavenging oxygen radical species in the liver, though the extract and kadsurin themselves may have an anti-oxidant property.

Keywords CCl_4-induced lipid peroxidation; *Kadsura heteroclita*; kadsurin; malondialdehyde; Schizandraceae; superoxide dismutase

Considerable studies have been focused on active oxygens and free radicals generated in the biological systems as causative agents, due to their close relation to toxicity, diseases and aging.[1] Active oxygens and free radicals induce lipid peroxidation in biological cell membranes, which subsequently leads to the production of a series of compounds including conjugated dienes, malondialdehyde, fluorescent substances *etc.* It has also been suggested that lipofuscin, an age-pigment, accumulates in the brain, heart and skin tissues through lipid peroxidation. Therefore, protection of membranes from abnormal lipid-peroxidation is essential for humans to maintain their health and to delay the aging processes.

In the course of our studies on the development of naturally-occurring anti-lipid peroxidative agents, we have reported that repeated administration of the extracts of pilose antler,[2-5] *Aegle marmelos*[6] and *Notopterygium* spp.[7] prevents CCl_4-induced lipid-peroxidation in the liver and the serum of mice.

In the present paper, we describe the anti-lipid peroxidative effects of an EtOH extract of the stems of *Kadsura heteroclita* (ROXB.) CRAIB. (Schizandraceae), which have been used for the treatment of gastric and duodenal ulcers, acute and chronic gastroenteritis, dysmenorrhea, postpartum abdominal pain, trauma and hepatic diseases in traditional Chinese medicine,[8] and of its major constituent, kadsurin,[9] using CCl_4-intoxicated mice.

Materials and Methods

Apparatus Ultra violet (UV) absorbance was measured with a Shimadzu UV-210 digital double beam spectrophotometer (Shimadzu Co., Kyoto, Japan). Fluorometric analysis was performed with a Shimadzu spectrofluorometer RF-5000.

Plant Material The stems of *Kadsura heteroclita* were collected in Feng-Qing county, Yunnan province of China, in August of 1989. The voucher specimens were deposited in the Herbarium of Materia Medica, China Pharmaceutical University, and in the Museum of Materia Medica, Toyama Medical and Pharmaceutical University.

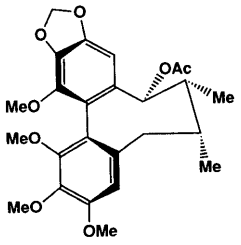

Chart 1. Structure of Kadsurin

Chemicals Thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), 1,1,3,3-tetrahydroxypropane, carbon tetrachloride (CCl_4), quinine sulfate, sulfosalicylic acid dihydrate (SAA), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and Tween 80 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Nitroblue tetrazolium (NBT) was purchased from Nacalai Tesque Co. (Kyoto, Japan), and xanthine and glutathione (reduced form) from Kohjin Co., Ltd. (Tokyo, Japan); xanthine oxidase (from buttermilk) and bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, U.S.A.); superoxide dismutase (SOD; 3300 unit/mg) from Toyobo Co., Ltd. (Osaka, Japan). Other chemicals were of a reagent grade. Kadsurin was isolated from the stems of *K. heteroclita* as reported previously.[9]

Preparation of a *K. heteroclita* Extract The powdered stems of *K. heteroclita* (100 g) were extracted five times with 95% EtOH (500 ml each) under 2 h reflux for each extraction. The combined EtOH solutions were evaporated *in vacuo* to give a residue in a yield of 3.1 g (3.1%).

Animals Male ddY mice weighing 18—20g (4 weeks) were used. All animals were maintained in conventional conditions at 23 ± 1 °C in an alternating 12 h light/dark cycle, fed a standard laboratory chow (CE-2, Clea Ltd., Tokyo, Japan) and were allowed water *ad. libitum*.

Administration of the *K. heteroclita* Extract Followed by CCl_4-Intoxication Five groups of ddY mice (5 mice each) were orally given the EtOH extract suspended in physiological saline containing 2% Tween-80 at doses of 0 (two groups), 75 (one group), 150 (one group) and 300 (one group) mg/kg/d, respectively, for 3 successive days. The animals were fasted 16 h after the last administration, and a mixture of CCl_4 and mineral oil (1 : 1, v/v) was then intraperitoneally administered to four groups at a dose of 60 μl/20 g body weight. One control group was given mineral oil only. Two hours after the CCl_4-intoxication, all mice were killed by decapitation, and the liver and blood were quickly sampled.

Administration of Kadsurin Followed by CCl_4-Intoxication Three groups of ddY mice (5 mice each) were orally given kadsurin suspended in the saline containing 2% Tween-80 at doses of 10, 20 and 40 mg/kg/d for three successive days, while two groups (5 mice each) were given the saline containing only 2% Tween-80. The animals except for one control group were intoxicated with CCl_4 as described above.

Determination of TBA-Reactive Substances (TBA-RS)[10] Two hundred milligrams of the liver were homogenized in an ice-cold 50 mM phosphate buffer, pH 7.4, to give a 10% (w/v) homogenate. After 10 min, 0.1 ml of 10% SDS, 2 ml of 0.1 N HCl and 1 ml of 1% TBA were added to a 0.1 ml portion of the liver homogenate. The mixture was heated for 45 min on a boiling water bath and extracted with 4 ml of BuOH after cooling. The BuOH phase was separated by centrifugation and the optical density was measured at 532 nm.

Determination of Lipid Conjugated-Dienes[11] Two hundred milligrams of the liver were homogenized in 2 ml of 0.3 M sucrose–3 mM ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.4. Four milliliters of $CHCl_3$–MeOH (2 : 1) were added to a 1 ml portion of the homogenate.

The mixture was shaken for 5 min and centrifuged at 1000 × *g* for 10 min. The organic layer was evaporated to give a residue by flashing with nitrogen gas. The residue was dissolved in cyclohexane to give a concentration of 1 mg/ml, and the lipid conjugated diene contents in the liver tissues of CCl_4-treated and untreated animals were compared by measuring the optical density at 275 nm. The difference in the conjugated dienes was expressed by $\Delta E^{1\%}_{cm}$ (the difference in absorbance between two samples in a 1% solution through a 1 cm path length).

Determination of Fluorescent Products[12] Six milliliters of a $CHCl_3$–MeOH (2 : 1) solution containing 200 mg of the liver tissues were homogenized for 1 min at 45 °C. A 3 ml portion of the homogenate was mixed with 3 ml of water and shaken for 5 min. The fluorescent products were determined by spectrofluorometry (excitation at 365 nm and emission at 440 nm).

Determination of SOD Activity According to the method of Geller and Winge,[13] the liver tissues were homogenized with a solution containing 0.25 M sucrose, 1 mM EDTA–Tris and 10 mM Tris–HCl, pH 7.4, with a Polytron homogenizer, and centrifuged at 300 × *g* for 10 min. SOD activity was determined by the method of Imanari *et al.*[14] A mixture containing 2.4 ml of 0.05 M carbonate buffer, pH 10.2, 0.1 ml of 3 mM xanthine, 0.1 ml of 0.75 mM NBT, 0.1 ml of 3 mM EDTA, 0.1 ml of 1.5 mg/ml BSA and 0.1 ml of the above test solution was preincubated for 15 min at 25 °C. The reaction was then started by adding 0.1 ml of xanthine oxidase and the mixture was incubated for 20 min at 25 °C. The reaction was stopped by adding 0.3 ml of 6 mM $CuCl_2$. The SOD activity was determined by measuring the absorbance at 560 nm. One unit of SOD activity was defined as the quantity of enzyme which causes 50% inhibition of NBT reduction under the above conditions.[14] Heat-labile SOD activity was determined by heating the test solution at 100 °C for 10, 20 and 30 min, followed by measuring the activity as described above.

Determination of Glutathione Content[15] The liver tissues were homogenized with ice-cold 50 mM phosphate buffer, pH 7.4, to give a 10% (w/v) homogenate. A 0.5 ml aliquot of the homogenate was added to 0.5 ml of 4% SAA, and the mixture was vigorously shaken and centrifuged at 1000 × *g* for 10 min. A part of the supernatant (0.5 ml) was mixed with 4.5 ml of DTNB and kept for 15 min at room temperature. The optical density at 412 nm was measured and the glutathione content was determined by using a calibration curve prepared with an authentic sample.

Determination of Protein Content The protein content was determined by the method of Lowry *et al.*[16] and the calibration curve was prepared with BSA.

Statistical Analysis The data is shown as mean ± S.E., and statistical significance was evaluated by the Dunnett test.

Results

For the purpose of investigating the effect of successive preadministration of a *K. heteroclita* extract and a major constituent, kadsurin,[9] on CCl_4-induced lipid peroxidation in mice, the contents of secondary and tertiary per-oxidation products such as TBA-RS, conjugated dienes, fluorescent lipid peroxidation products and glutathione, as well as SOD activity in the liver tissues, were determined according to the standard methods as described in the preceding papers.[6,7]

TBA-RS in the Liver Tissue and Serum Intraperitoneal administration of CCl_4 to mice prior to decapitation resulted in a significant increase of TBA-RS in the liver tissue and the serum, the levels being 1.7-2 fold higher than those of a non-administered group (Table I). Three days of successive *p.o.* administration of the *K. heteroclita* extract followed by CCl_4-intoxication led to reduce the formation of TBA-RS in the liver tissue and the serum. At a dose of 300 mg/kg/d, the TBA-RS levels in the liver tissue and the serum were restored to the respective normal levels. Similarly, successive preadministration of kadsurin, a major constituent of the stems of *K. heteroclita*, at doses of 20—40 mg/kg/d reduced the TBA-RS levels, though no appreciable effect was observed at a dose of 10 mg/kg/d.

Lipid Conjugated Dienes in the Liver Tissue According to the method of Recknagel and Glende,[11] lipid extracts from the liver tissues of mice treated with or without the *K. heteroclita* extract were prepared and their absorbances at 275 nm, mostly due to the presence of conjugated-dienes produced by lipid peroxidation, were measured. The levels of lipid conjugated-dienes induced by CCl_4-intoxication diminished significantly in a dose-dependent manner as shown in Table II. Successive preadministration of kadsurin also reduced the formation of the conjugated-dienes at smaller doses.

Fluorescent Lipid-Peroxidation Products in the Liver Tissue By intoxication with CCl_4, fluorescent products, possibly formed from malondialdehyde (MDA) and tissue components, significantly increased in the liver tissues of mice when monitored by fluorescent intensity (Table III). However, successive preadministration at doses of 75—300 mg/kg/d of the *K. heteroclita* extract or at doses of 20—40 mg/kg/d of kadsurin led to a significant decrease in fluorescent intensity, the levels of the fluorescent products being close to that of a normal control.

SOD Activity in the Liver Tissue Table IV shows the

Table I. Effect of Preadministration of the *K. heteroclita* Extract (KHE) and Kadsurin on TBA-RS Formation in the Liver and the Serum of Mice Intoxicated with CCl_4

Treatment	Dose (mg/kg/d)	CCl_4-intoxication	TBA-RS	
			Liver (nmol/100 g wet wt.)	Serum (nmol/ml)
Normal control		−	32.3 ± 0.65[a]	30.7 ± 0.39[a]
CCl_4-treated control		+	66.2 ± 2.98	53.6 ± 1.24
+ KHE	75	+	54.0 ± 2.45[a]	44.5 ± 3.05[b]
+ KHE	150	+	37.8 ± 1.41[a]	34.0 ± 0.94[a]
+ KHE	300	+	35.2 ± 1.18[a]	30.8 ± 2.08[a]
+ Kadsurin	10	+	62.9 ± 2.40	45.8 ± 3.28[b]
+ Kadsurin	20	+	55.3 ± 1.86[a]	36.5 ± 1.48[a]
+ Kadsurin	40	+	34.6 ± 1.01[a]	32.4 ± 1.36[a]

Values are means ± S.E. of 5 determinations. A mixture of CCl_4 and mineral oil (1 : 1) was intraperitoneally administered at a dose of 60 μl/20 g body weight 2 h before decapitation. Statistical significance compared to a CCl_4-intoxicated group: *a*) $p < 0.01$; *b*) $p < 0.05$.

Table II. Effect of Preadministration of the *K. heteroclita* Extract (KHE) and Kadsurin on the Formation of Lipid Conjugated Dienes in the Liver Tissues of Mice Intoxicated with CCl_4

Treatment	Dose (mg/kg/d)	CCl_4-intoxication	Lipid conjugated diene
			[$\Delta E^{1\%}_{cm} \times 10$/100 mg wet wt.]
CCl_4-treated control		+	4.18 ± 0.35
+ KHE	75	+	2.25 ± 0.13[a]
+ KHE	150	+	0.87 ± 0.13[a]
+ KHE	300	+	0.18 ± 1.49[a]
+ Kadsurin	10	+	2.79 ± 2.86[a]
+ Kadsurin	20	+	1.73 ± 1.58[a]
+ Kadsurin	40	+	1.20 ± 2.18[a]

The values indicate differences in optical density at 275 nm for lipids prepared from liver homogenates of CCl_4-treated groups and an untreated one, and are expressed as means ± S.E. of 5 determinations. A mixture of CCl_4 and mineral oil (1 : 1) was intraperitoneally administered at a dose of 60 μl/20 g body weight 2 h before decapitation. Statistical significance compared to a CCl_4-intoxicated group: *a*) $p < 0.01$.

Table III. Effect of Preadministration of the *K. heteroclita* Extract (KHE) and Kadsurin on the Formation of Fluorescent Products in the Liver Tissues of Mice Intoxicated with CCl_4

Treatment	Dose (mg/kg/d)	CCl_4-intoxication	Fluorescent products: Fluorescence unit/100 mg wet wt.
Normal control		−	71.8±1.65[a]
CCl_4-treated control		+	98.5±1.62
+KHE	75	+	73.4±1.95[a]
+KHE	150	+	69.5±1.38[a]
+KHE	300	+	67.1±1.49[a]
+Kadsurin	10	+	81.9±2.86[a]
+Kadsurin	20	+	72.6±1.58[a]
+Kadsurin	40	+	68.5±2.18[a]

Values are means±S.E. of 5 determinations. A mixture of CCl_4 and mineral oil (1 : 1) was intraperitoneally administered at a dose of 60 μl/20 g body weight 2 h before decapitation. Statistical significance compared to a CCl_4-intoxicated group: *a*) $p<0.01$.

Table IV. Effect of Preadministration of the *K. heteroclita* Extract (KHE) and Kadsurin on SOD Activities in the Liver Tissues of Mice Intoxicated with CCl_4

Treatment	Dose (mg/kg/d)	CCl_4-intoxication	Specific activity [Units/100 mg wet wt.]
Normal control		−	17.1±0.67[a]
CCl_4-treated control		+	5.1±0.45
+KHE	75	+	7.3±0.13
+KHE	150	+	18.0±0.49[a]
+KHE	300	+	19.2±0.49[a]
+Kadsurin	10	+	6.4±0.40
+Kadsurin	20	+	17.0±1.39[a]
+Kadsurin	40	+	19.0±1.52[a]

Values are means±S.E. of 5 determinations. A mixture of CCl_4 and mineral oil (1 : 1) was intraperitoneally administered at a dose of 60 μl/20 g body weight 2 h before decapitation. Statistical significance compared to a CCl_4-intoxicated group: *a*) $p<0.01$.

SOD activity in the liver tissues, determined by the method of Imanari *et al.*[14] The SOD-like activity in the liver tissues of the mice treated with CCl_4 decreased to approx. one-third that of a normal control (CCl_4-untreated group). Successive preadministration of the *K. heteroclita* extract (75—300 mg/kg/d) or of kadsurin (20—40 mg/kg/d) resulted in a significant increase in the SOD activity reduced by CCl_4-intoxication, the activity being recovered up to the level of the normal control group. Preincubation of the liver homogenate at 100 °C led to the time-dependent loss in SOD activity, suggesting that heat-labile enzymes are responsible for the activity (Fig. 1).

Glutathione Level in the Liver Tissue Table V shows the glutathione levels in the liver tissues of mice with or without CCl_4-intoxication. Contrary to the above biochemical indices of lipid peroxidation, the glutathione levels did not significantly change between the liver tissues of the normal control group and the CCl_4-intoxicated control group. Successive pretreatments with either the *K. heteroclita* extract at doses of 75—300 mg/kg/d or kadsurin at doses of 10—20 mg/kg/d did not alter the glutathione level. However, the glutathione level was significantly lowered with pretreatment with kadsurin at a dose of 40 mg/kg/d.

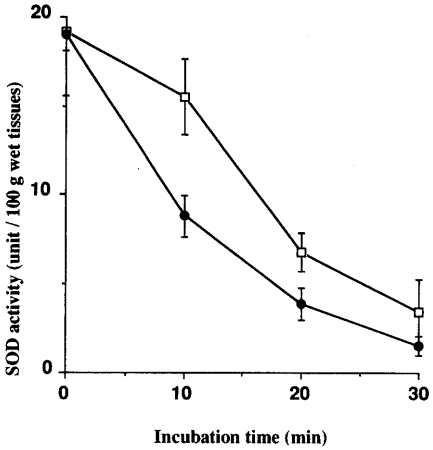

Fig. 1. Thermal Stability of SOD in the Liver Homogenate

The homogenates prepared from the livers of mice which had been successively given the *K. heteroclita* extract at a dose of 300 mg/kg/d (□) and kadsurin at a dose of 40 mg/kg/d (●) were incubated at 100 °C for indicated periods and their SOD activities were determined by the method of Imanari *et al.*[14]

Table V. Effect of Preadministration of the *K. heteroclita* Extract (KHE) and Kadsurin on Glutathione Levels in the Liver Tissues of Mice Intoxicated with CCl_4

Treatment	Dose (mg/kg/d)	CCl_4-intoxication	Glutathione [μmol/g wet wt.]
Normal control		−	5.3±0.13
CCl_4-treated control		+	5.8±0.22
+KHE	75	+	6.0±0.27
+KHE	150	+	5.3±0.36
+KHE	300	+	4.8±0.31
+Kadsurin	10	+	6.5±0.63
+Kadsurin	20	+	5.4±0.18
+Kadsurin	40	+	4.5±0.36[a]

Values are means±S.E. of 5 determinations. A mixture of CCl_4 and mineral oil (1 : 1) was intraperitoneally administered at a dose of 60 μl/20 g body weight 2 h before decapitation. Statistical significance compared to a CCl_4-intoxicated group: *a*) $p<0.05$.

Discussion

Administration of CCl_4 to animals results in the generation of free radicals such as $\cdot CCl_3$ and $\cdot CClO$ in the liver by reduced nicotinamide adenine dinuclotide (NADPH)–cytochrome P-450, which leads to lipid peroxidation. The lipid peroxidation is monitored with increased levels of TBA-RS, lipid conjugated-dienes and fluorescent products[12] which are formed through chain reactions induced by free radicals on the cell membrane. In the present experiments, we have demonstrated that 3 d successive *p.o.* administration of a *K. heteroclita* extract to mice appreciably prevents the lipid peroxidation induced by CCl_4-intoxication. Since the stems of *K. heteroclita* contained various lignans having a dibenzocyclo-octadiene skeleton,[9] we examined the anti-lipid peroxidative effect of kadsurin as representative of this type of lignans and found that it also prevented the CCl_4-induced lipid peroxidation but at even smaller doses.

As reported in the previous papers,[9] we isolated seven dibenzocyclo-octadiene type lignans, such as kadsurin, interiorin and heteroclitins A—E, from the stems of *K. heteroclita*. These compounds have more or less antioxidant properties,[17] but kadsurin seems to be the major constituent responsible for the anti-lipid peroxidative action of the *K.*

heteroclita extract, due to its high content in the extract. Maeda *et al.*[18] reported that pretreatment of gomisin A, a dibenzocyclo-octadiene type lignan from the schizandra fruit, appreciably reduced the activities of serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and lactate dehydrogenase (LDH) elevated by CCl_4-intoxication to rats, and inhibited hepatocellular necrosis, showing a protecting action of the lignan against liver injury by CCl_4. Furthermore, Kiso *et al.*[19] reported that benzocyclo-octadiene type lignans, wuweizisu C and gomisin A, inhibited CCl_4-, adenosine diphosphate (ADP)/Fe^{3+}- and ascorbate/Fe^{2+}-induced lipid peroxidation, though these compounds showed no direct inhibition in CCl_3 radical formation in the presence of rat liver microsomes. This data suggests that dibenzocyclo-octadiene type lignans protect the liver injury by inhibiting a non-enzymatic step in the processes of lipid peroxidation. However, in our experiments, SOD activity in the liver tissue was appreciably elevated by successive *p.o.* preadministration of the *K. heteroclita* extract or kadsurin, suggesting another possibility: that they induce SOD enzyme(s) and/or activate the enzyme activity in the liver tissue. Since repeated administration of the extract of *N. incisum* or *A. marmelos*, which showed a similar anti-lipid peroxidative activity in the CCl_4-intoxicated mice,[6,7] resulted in the induction of SOD-like enzyme(s),[20] the *K. heteroclita* extract and its constituent, kadsurin, seem to induce radical scavenging enzyme(s) in a similar manner.

Lu and Liu[21] observed that dibenzocyclo-octadiene type lignans, schisanhenol and schizandrin, inhibited vitamin C/NADPH induced lipid peroxidation and gossypol-induced super oxide generation *in vitro*, and increased SOD and catalase activities in rat liver cytosol by successive oral administration, which led them to conclude that these lignans not only inhibit microsomal lipid peroxidation by scavenging active oxygen radicals, but also enhance the ability of the body to eliminate active oxygen species by inducing SOD and catalase activities. Our present results support their proposed mechanisms of anti-lipid peroxidative action for dibenzocyclo-octadiene type lignans.

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