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Antioxidative and Superoxide Scavenging Activities of Retrochalcones in *Glycyrrhiza inflata*

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Abstract—Licochalcone A, B, C, D and echinatin, retrochalcones isolated from the roots of *Glycyrrhiza inflata* (licorice), along with an ordinary chalcone isoliquiritigenin, were assessed for their inhibitory activities on lipid peroxidation in various systems and radical scavenging activity. Among those tested, licochalcones B and D strongly inhibited superoxide anion production in the xanthine/xanthine oxidase system. These two compounds also showed potent scavenging activity on DPPH radical. Microsomal lipid peroxidation induced by Fe(III)-ADP/NADPH was inhibited almost completely by 3 μg/ml of licochalcones B and D. Mitochondrial lipid peroxidation induced by Fe(III)-ADP/NADH was more sensitive to these retrochalcones; almost complete inhibition was observed at 10 μg/ml of all retorochalcones tested. Licochalcones B and D scavenged superoxide anion in microsome. Furthermore, these retrochalcones protected red cells against oxidative hemolysis. These phenolic compounds were shown to be effective to protect biological systems against various oxidative stresses.

Introduction

There has been increasing evidence that the inadvertent overproduction of active oxygen species may result in oxidative tissue injury overcoming the protective defense mechanisms against oxidants.[1] Active oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxy radical (OH·), are formed through a one-electron reduction process of molecular oxygen (O_2). They are generated by a process known as redox cycling, and are catalyzed by transition metals, such as Fe^{2+} and Cu^+ to cause DNA damage, thiol oxidation and lipid peroxidation that can lead to cell death.[2] They are also considered to be implicated in some diseases such as cardiovascular disorder, chronic gut inflammation, cancer and AIDS.[3,4]

It is well established that lipid peroxidation is one of the reactions set into motion as a consequence of the formation of free radicals in cells and tissues. Lipid peroxidation is initiated by active oxygen species attacking unsaturated fatty acids, and is propagated by a chain reaction cycle involving lipids, peroxy radicals and lipid hydroperoxides.[5] Membrane lipids are particularly susceptible to peroxidation not only because of the high content of polyunsaturated fatty acids but also because of their association in the cellular membrane with enzymatic and non-enzymatic systems capable of generating free radical species.[6] Since unsaturated fatty acids are most important components of biological membranes and impart desirable properties upon the fluidity of cellular membrane structure, the peroxidation of unsaturated fatty acids in biological membranes leads to disruption of membrane structure and function.[7,8] This kind of degradative process is implicated in several pathologic conditions including serious derangements such as ischemia-reperfusion injury,[9] coronary arteriosclerosis[10] and diabetes mellitus[9] as well as associated with aging and carcinogenesis.[11] The propagation cycle of lipid peroxidation is broken by either enzymatic inactivation of reactive oxygen species involved in the chain reaction or non-enzymatic reactions due to the intervention of free radical scavengers and antioxidants.[12–14] Some antioxidants have been reported to

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be effective in prevention cancer and coronary heart disease[15] and the protection of myocardium from experimental myocardial infraction,[16] and effective as a prophylactic agent against neuronal symptoms resulting from aging.[17] A number of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been developed so far, but their use as therapetic agents has been hampered by their toxicity.[18,19] Vitamine E (α-tocopherol) is also an effective naturally occurring antioxidant, but has yet to find wide use.[20] Therefore, more effective antioxidants with less toxicity and minimal side effects, in particular those derived from natural source such as folk medicines and food, have been desired not only as therapeutic agents in the field of preventive medicine but also as additives in the food industry.[21–23]

Licorice is an esteemed crude drug in both the Orient and Occident that is originated from the dried roots of several *Glycyrrhiza* species. It has still found medicinal value because of its wide-ranging therapeutic properties including relief of rheumatic and other types of pain and healing effect on ulcers.[24] The crude extract of licorice has also found commercial use as food additive in Japan since it contains the sweetening principle glycyrrhizin. Recent chemical investigations have revealed the presence of a wide variety of bioactive phenolic constituents in licorice,[25] which have drawn attention as a potential source of chemical leads. *G. inflata* is one of the main botanical source of licorice, and is chemically characterized by the presence of 'retrochalcones', which are distinguished from ordinary chalcones by the absence of oxygen-functionality at the 2-position.[26] Five retrochalcones, licochalcone A–D and echinatin (Figure 1), have been isolated and characterized from commercially available *G. inflata* root.[26] Two retrochalcones, licochalcone A and B, were reported to be effective as additives to suppress autoxidation in lard.[27] This paper is to assess the inhibitory activity of these five retrochalcones on lipid peroxidation mediated by reactive oxygen species and their radical scavenging effect in various assay systems, and to further look into the structure–activity relationship of chalcones for these activities.

Results

In the present study, the antiperoxidative activities of licorice chalcones were assessed using the different assay systems; (1) scavenging activity of free radicals based on chemical trapping; (2) inhibitory activity on superoxide

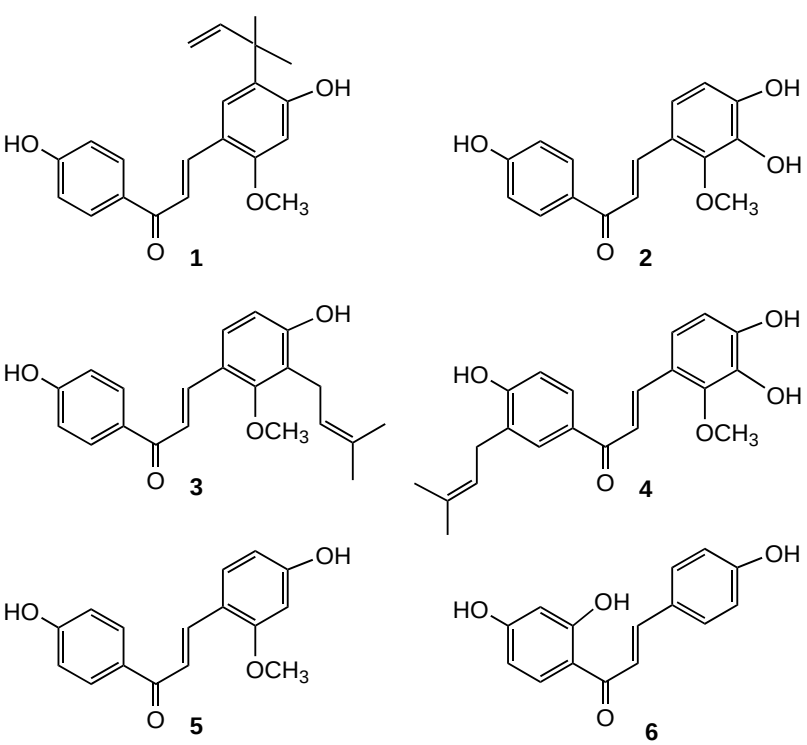

Figure 1. Structure of retrochalcones isolated from *Glycyrrhiza inflata* and an ordinary chalcone isoliquiritigenin. **1**, licochalcone A; **2**, licolchalcone B; **3**, licochalcone C; **4**, licochalcone D; **5**, echinatin; **6**, isoliquiritigenin.

anion generation by both the xanthine–xanthine oxidase system and rat liver microsomes; (3) inhibitory activity on lipid peroxidation in rat liver microsomes and mitochondria; and (4) protective activity of red blood cells from peroxidation-induced hemolysis. An ordinary chalcone widely occurring in licorice, isoliquiritigenin (Figure 1), was also subjected to the above assay along with retrochalcones.

Free radical scavenging activity of chalcones was evaluated by the colorimetrical decrease in absorbance of diphenyl-*p*-picrylhydrazyl (DPPH) due to the chemical trapping of unpaired electron, and the results were given in Figure 2 as dose-response curves. Licochalcone B and D were potent free radical scavengers.

Superoxide anion is produced by several oxidative enzymes as a product in the one-electron reduction of oxygen.[2] Xanthine oxidase is one of the major oxidative enzymes producing superoxide anion, resulting in tissue injury.[28] The generation of superoxide anion by the xanthine-xanthine oxidase system was effectively inhibited by chalcones in a dose-dependent manner as shown in Figure 3. Licochalcone B and D exhibited especially potent activity, and their IC_{50} values were observed at 2.3 and 4.0 μg/ml, respectively. The reduction of nitroblue tetrazolium by superoxide anion generated by xanthine oxidase was inhibited almost 80% at 10 μg/ml of these chalcones. The direct inhibition on enzyme activity of xanthine oxidase at this concentration of licochalcone B and D was less than 30%. The cellular sourses of superoxide anion in mammalian cells include the microsomal electron transfer chain, entailing a slow electron transfer to O_2 via NADPH-cytochrome P-450 and NADPH-cytochrome b_5 reductase.[29] Superoxide anion produced on incubation of liver microsomes with NADPH can be detected by reduction of succinoylated ferricytochrome *c*. The addition of excessive superoxide dismutase (SOD) enables superoxide anion-mediated reduction of cytochrome *c* to be distinguished from its direct enzymatic reduction, since reduction of modified cytochrome *c* is inhibited by SOD. As shown in Figure 4, licochalcone B strongly inhibited reduction of modified ferricytochrome *c* after 3 min incubation at a dose of 30 μg/ml. Licochalcone D also showed superoxide anion scavenging effect [Figure 4(b)], but not as potent as licochalcone B. Significant activity was not observed with licochalcone A, C and echinatin. Superoxide sacvenging activity of licochalcone B and D in xanthine oxidase system was similar degree, however, licochalcone B showed potent effect than licochalcone D in microsomes. Cytochrome b_5 reductase activity was a little affected by these chalcones, and the inhibition by licochalcone B was potent than that by licochalcone D. The difference in radical scavenging activity among DPPH radical, xanthine oxidase system and microsomal electron transport system would be caused by the direct action on enzyme activities.

Microsomes, especially smooth surfaced endoplasmic reticulum, are liable to producing lipid peroxides, and are considered to provide other tissues with peroxides formed to cause hemolysis.[30] NADPH–cytochrome P-450 reductase and cytochrome P-450 are involved in

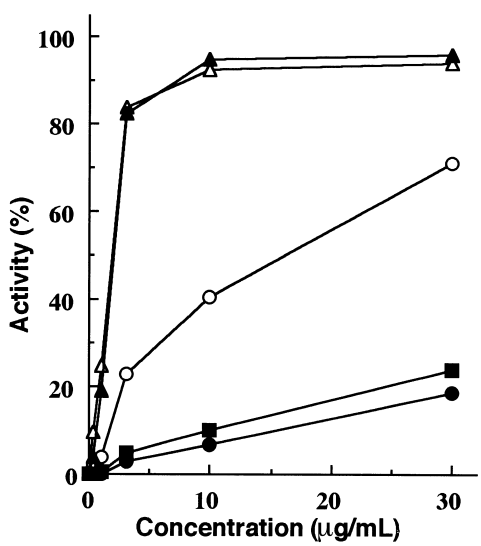

Figure 2. Scavenging activity of retrochalcones on DPPH radical. Each plot is the mean of triplicate determinations. (○) **1**, (△) **2**, (●) **3**, (▲) **4**, (■) **5**.

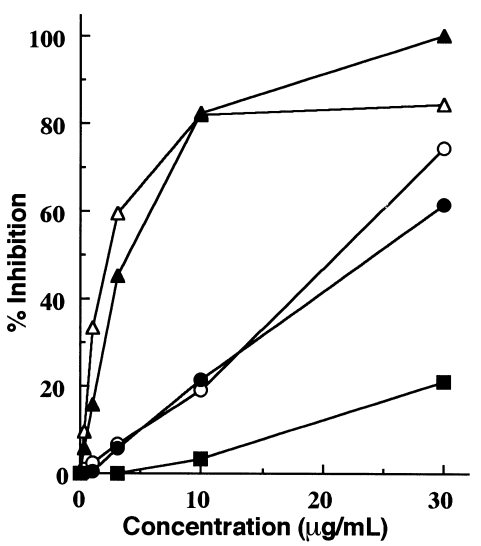

Figure 3. Inhibitory effects of retrochalcones on the generation of superoxide anion. Each plot is the mean of triplicate determinations. (○) **1**, (△) **2**, (●) **3**, (▲) **4**, (■) **5**.

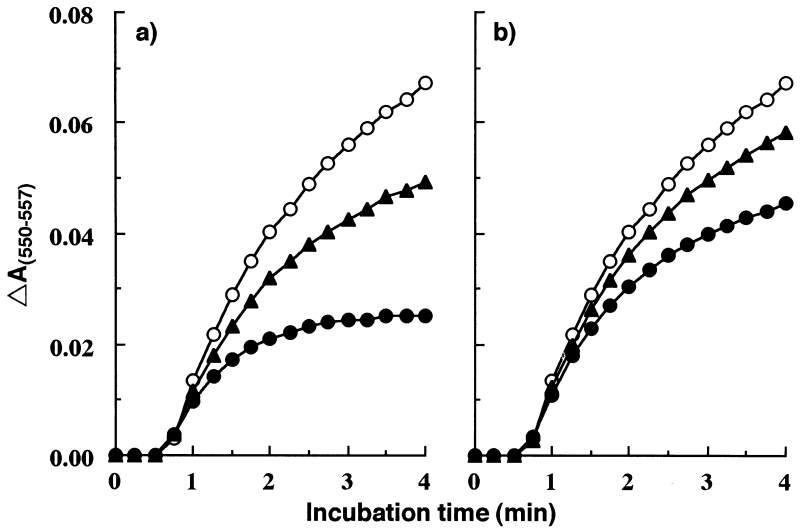

Figure 4. Superoxide radical scavenging activity of licochalcone B and D in microsome. (○) control, (▲) 10 μg/ml, (●) 30 μg/ml. (a) Licochalcone B, (b) licochalcone D.

microsomal lipid peroxidation that can be induced by Fe^{3+}–ADP/NADPH.[31] The extent of lipid peroxidation thus induced can be measured by the thiobarubituric acid (TBA) method. The antiperoxidative activity of retrochalcones was assessed by this method, and the results are shown in Figure 5. Licochalcone B and D were especially effective to prevent the microsomal lipid peroxidation with a minimum dose of complete inhibition put at 3 μg/ml, whereas a synthetic antioxidant butylated hydroxytoluene (BHT) required 10 μg/ml for complete inhibition. At this concentration of these licochalcones, the direct inhibition on cytochrome P-450 reductase was less than 30%. As in the case of microsomes, mitochondria are also constantly exposed to oxidative stress since they are major cellular organs for redox reactions.[32] In non-damaged mitochondrial respiratory chain where the four electrons reduction of oxygen occures to produce water, the leak of one electron occurs at some sites of the chain and leads to formation of superoxide anion and hydrogen peroxide, which cause mitochondrial lipid peroxidation.[33,34] By adding rotenone, a specific inhibitor of the electron transport system, to the assay system, mitochondrial lipid peroxidation can be induced more effectively on incubation of submitochondrial particles with Fe^{3+}–ADP/NADH. Mitochondrial lipid peroxidation was more sensitive to inhibition by licochalcones than microsomal peroxidation; licochalcone A and D completely inhibited mitochondrial lipid peroxidation at a dose of 1 μg/ml (Figure 6). When the direct actions of these chalcones on mitochondrial electron transport system were measured, the inhibition of NADH oxidase and NADH-cytochrome c reductase activities was less than 10% by 1 μg/ml of licochalcone A and D.

The red blood cells are intrinsically exposed to a higher oxygen tension as compared to all other tissues, and are left vulnerable to oxidative process due particularly to the presence of hemoglobin that is one of the most powerful catalysts capable of initiating lipid peroxidation.[35] Erythrocyte hemolysis can be induced easily by intracellular generation of oxygen free radicals as a result of membrane damage via lipid peroxidation. The inhibitory effects of retrochalcones on hemolysis were tested utilizing an azo-initiator 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH) as an intracellular

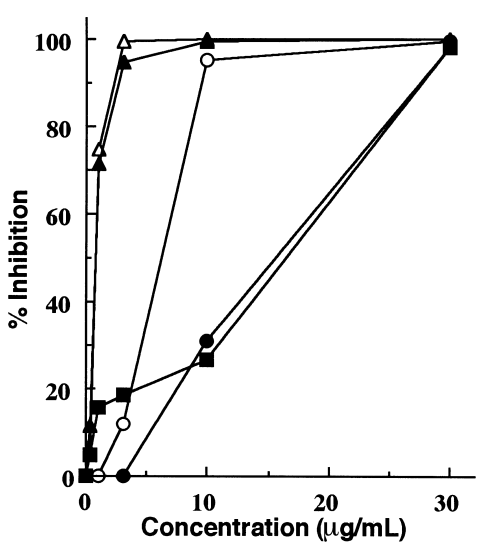

Figure 5. Effects of retrochalcones on rat liver microsomal lipid peroxidation. Each plot is the mean of triplicate determinations. (○) **1**, (△) **2**, (●) **3**, (▲) **4**, (■) **5**.

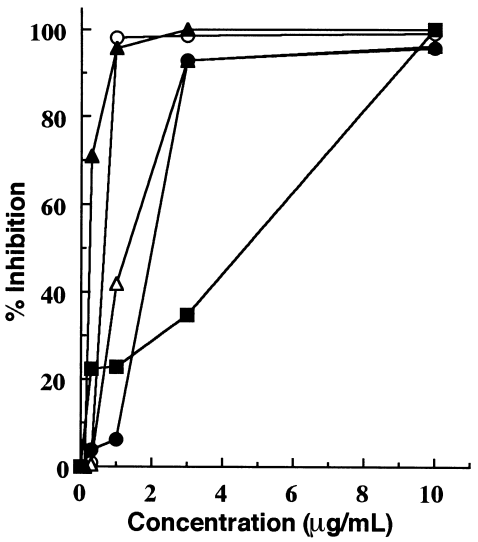

Figure 6. Effects of retrochalcones on rat liver mitochondrial lipid peroxidation. Each plot is the mean of triplicate determinations. (○) **1**, (△) **2**, (●) **3**, (▲) **4**, (■) **5**.

generator of oxygen free radicals in human erythrocytes.[36] As shown in Figure 7, AAPH-induced erythrocyte hemolysis was prevented effectively by licochalcones B and D (30 and 36% hemolysis observed, respectively) due to an effect ascribed to the antioxidative properties of retrochalcones, while 85% of erythrocytes were damaged on incubation without retrochalcones in the presence of AAPH.

Discussion

Chalcones are a group of phenolic compounds belonging to the flavonoid family, and widely occur in nature as pigments. Though such flavonoids as flavones and flavonols are known to exhibit a wide variety of biological effects including antioxidant activity and have drawn attention as possible therapeutic agents,[37] there have been only a few reports referring to biological activity of chalcones. In this paper the authors have proved that chalcones, obtained from *G. inflata* root, are effective antioxidants and radical scavengers not only suppressing lipid peroxidation in several biological systems but also protecting erythrocytes from hemolysis induced by oxygen free radicals as mentioned above.

Lipid peroxidation is a complex process, and the extent of peroxidation is variably dependent upon the initiation mechanism, which can be either enzymatic or non-enzymatic. What are termed antioxidants can influence the peroxidation process through either a simple or complex mechanisms including free radical scavenging, divalent metal chelation and so on.

Iron ion-dependent lipid peroxidation will be affected by substrates capable of metal chelation, and the inhibitory activity shown by some flavones possessing either an *ortho*-dihydroxy, 3- or 5-hydroxy group was ascribed to this mechanism.[38–40] Since licochalcones B, D, and isoliquiritigenin possess either a carbonyl chelated hydroxy or ortho-dihydroxy group, a part of their antiperoxidative activity will be based on the same mechanism.

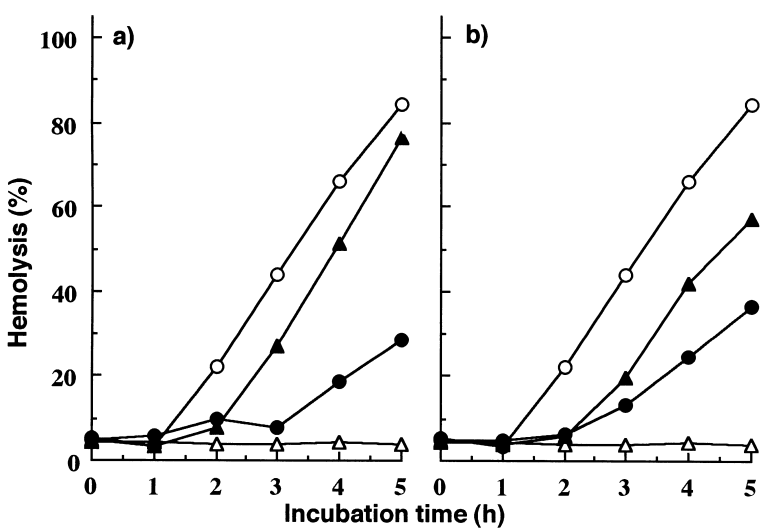

Figure 7. Inhibition of oxidative hemolysis by licochalcone B and D in human erythrocytes. Each plot is the mean of triplicate determinations. (○) control, (▲) 10 μg/ml, (●) 30 μgml, (△) without AAPH. (a) Licochalcone B, (b) licochalcone D.

Phenolic compounds with free hydroxyls as represented by flavonoids are considered to possess, more or less, free radical scavenger activity, since phenoxy radicals are readily formed by donation of phenolic hydrogens and subsequently react with oxygen free radicals involved in lipid peroxidation. In general, the phenoxy radical can be stabilized by its delocalization in the conjugate chromophore system. The chalcone skeleton has an elongated conjugate system, and the magnitude of conjugation will be enhanced effectively by the introduction of electron donating groups in the B-ring as is apparently indicated by their UV specral features.[41] This will be in part the basis for the significant antioxidative activity of licochalcone A, C, and echinatin, all of which lack no chelating hydroxyl but possess a strong electron donating methoxyl at 2-position.

The catechol is generally very sensitive to oxidation, especially the one mediated by free radicals, and the formation of relatively stable *ortho*-semiquinone radical through donation of catechol hydrogen can be ascribed. The most part of potent antiperoxidative activity exhibited by licochalcone B and D would be based on this mechanism, though the *ortho*-dihydroxy group (catechol moiety) can disturb lipid peroxidation by the trapping of iron ion, an essential cofactor of peroxidation process, through its divalent chelation ability. One of the earlier works pointed out that the presence of this functionality is necessary for inhibitory activity on nonenzymic peroxidation by flavones.[38] In reality, the most potent activity was observed in compounds having a free catechol group at 3′- and 4′-position of flavone skeleton.[39] In the case of licochalcones B and D where an *ortho*-dihydroxy group occurs in B-ring, their antiperoxidative activity strikingly increased by one order of magnitude as compared to other chalcones lacking such functional group (echinatin versus licochalcones B and D). The presence of a methoxyl *ortho* to 3′,4′-dihydroxy in these two retrochalcones will most contribute to increases in antiperoxidative activity, since such strong electron-donating group would further stabilize rather stable *ortho*-semiquinone radicals. These structural features will be the basis for potent inhibitory activity of licochalcones B and D on lipid peroxidation and radical scavenging activity, which was more than equivalent to that of a synthetic antioxidant BHT.

Some naturally occurring polyhydroxyflavones have been proved to be effective inhibitors of lipid peroxidation in cell free systems.[39] However, their potential efficiency as therapeutic agents will be in doubt since they are not hydrophobic enough to penetrate into the cell and reach tissues where they are expected to inactivate free radicals involved in some diseases. Chalcones tested for their antiperoxidative activities in the present study are more hydrophobic than polyhydroxyflavones such as quercetin and so on. In particular, licochalcones A, C and D have prenyl groups (C_5 units) in either A- or B-ring. The introduction of such lipophylic substitution into these molecules will increase their hydrophobicity, which is expected to help improve their biochemical and pharmacological properties through enhanced affinity for the plasma membrane. The effect of such substitution is best demonstrated by much enhanced antiperoxidative activities of licochalcones A and C as compared to those of echinatin and significant increase in activity of licochalcones D versus B.

Experimental

Licochalcones A, B, C, D and echinatin were isolated from the roots of *Glycyrrhiza inflata*.[26] Xanthine oxidase, butylated hydroxytoluene (BHT), thiobarubituric acid (TBA), ADP, superoxide dismutase (SOD) and cytochrome *c* were purchased from Sigma Chemical Co. (St. Louis, MO). NADH and NADPH were obtaind from Oriental Yeast Co. (Tokyo, Japan). Diphenyl-*p*-picrylhydrazyl (DPPH) and 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Succinoylated ferricytochrome *c* was prepared according to the method described by Kuthan and Ullrich.[42]

Assay for radical scavenging activity on DPPH

The reaction mixture consisted of 1 mL of 100 mM acetate buffer (pH 5.5), 1 mL of ethanol and 0.5 mL of 2.5 mM ethanolic solution of diphenyl-*p*-picrylhydrazyl (DPPH). After allowing the mixture to stand at room temperature for 20 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity of each sample was expressed as percentage of a decrease in absorbance of DPPH against that of a control DPPH solution.[43]

Assay for inhibitory activity of superoxide anion by xanthine oxidase

The raction mixture consisted of 40 mM sodium carbonate buffer (pH 10.2) containing 0.1 mM xanthine, 0.1 mM EDTA, 50 μg protein/ml of bovine serum albumin, 25 mM nitroblue tetrazolium, and 3.3×10^{-3} units xanthine oxidase (XOD, EC 1.2.3.2) in a final volume of 3 mL. After incubation at 25°C for 20 min, the reaction was terminated by the addition of 0.1 mL of 6 mM $CuCl_2$. The absorbance of formazan produced was determined at 560 nm, and the inhibitory activity of each sample on superoxide anion generation was estimated.[44] For studies of xanthine oxidase activity, the rise in the absorbance at 290 nm due to urate production was measured in the absence of nitrobleu tetrazolium.[45]

Preparation of mitochondria and microsomes

Livers of Wistar male rat weighing 100–150 g were removed quickly and soaked into ice-cold 3 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA. Mitochondria were obtained as a pellet after centrifugation at 15,000 *g* according to the method of Johnson and Lardy,[46] and then resuspended in 100 mM HEPES buffer (pH 7.2). Submitochondrial particles were prepared by sonication[47] of mitochondrial suspension for 1 min at 4 °C using a Model 450 Sonifier (Branson Ultrasonics Corporation, USA). Microsomes were obtained as a pellet after centrifugation at 105,000 *g* for 60 min,[48] and then the pellet was resuspended in the buffer containing 70 mM sucrose, 0.21 M mannitol, 0.1 mM EDTA and 3 mM HEPES (pH 7.4). Protein concentrations of the suspensions were determined by the method of Lowry et al.[49]

Assay for inhibitory activity on generation of superoxide anion in microsomes

Rat liver microsomes were diluted to a final concentration of 0.4 mg/mL in 0.1 M Tris buffer (pH 7.7) containing 0.1 mM EDTA and 30 μM succinoylated ferricytochrome *c*.[42] The reaction mixtures were incubated at 37°C, and after 30 s, NADPH was added to the final concentration of 0.2 mM. The reduction of succinoylated cytochrome *c* was monitored using the wavelength-pair 550 minus 557 nm[42] using a MPS-2000 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with a TCC temperature controller. The O_2^- mediated reduction of cytochrome *c* was discriminately detected by the addition of of an excess of SOD that suppresses the direct enzymatic reduction of cytochrome *c*. Superoxide anion generated in the above system in the presence or absence of each sample was quantified according to the method reported in the literature,[42] and then its inhibitory activity on microsomal superoxide anion generation was estimated.

Assay for lipid peroxidation in microsomes and mitochondria

The NADPH-dependent peroxidation of microsomal lipid was assayed by the modified method described by Pederson et al.[31] Rat liver microsomes (equivalent 0.2 mg protein) were incubated at 37 °C in 1 mL of reaction mixture containing 0.05 M Tris-HCl (pH 7.5), 2 mM ADP, and 0.12 mM $Fe(NO_3)_3$. The reaction was started by the addition of 0.1 mM NADPH, and stopped after 5 min by the addition of 2 mL of TCA–TBA–HCl reagent (15% w/v trichloroacetic acid; 0.375% thiobarbituric acid; 0.25 N HCl) and 90 μL of 2% BHT solution. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1,000 *g* for 10 min. The malondialdehyde contents of the supernatant as a TBA conjugate was calculated from the absorbance at 535 nm by use of an extinction coefficient of 1.56×10^5 $M^{-1}\,cm^{-1}$.[50]

Mitochondrial lipid peroxidation was assayed according to modification of the method described by Takayanagi et al.[51] Rat liver submitochondrial particles (equivalent 0.3 mg protein) were incubated at 37 °C in 1 mL of a reaction mixture containing 50 mM HEPES-NaOH (pH 7.0), 2 mM ADP, 0.1 mM $FeCl_3$, and 10 μM rotenone. The reaction was initiated by the addition of 0.1 mM NADH, and terminated after 5 min. The formation of malondialdehyde was determined by the TBA method as described above.

Assay for enzyme activity in microsomes and mitochondria

Cytochrome P-450 reductase activity in microsomes was measured in 40 mM potassium phosphate buffer (pH 7.5) containing 10 mM NaN_3, 1 mg/mL of cytochrome *c*, 0.1 mM NADPH and microsomal suspension. The reaction was initiated by the addition of NADPH, and the reduction of cytochrome *c* was monitored at 550 nm at 37 °C.[52]

Microsomal cytochrome b_5 reductase activity was assayed as NADH ferricyanide reductase activity.[53] The reaction was carried out in 0.1 M potassium phosphate buffer (pH 7.5), 1 mM potassium ferricyanide, 0.1 mM NADH and microsomal preparation. The reaction was started by the addition of NADH, and the reduction of ferricyanide was followed at 420 nm.[53]

Mitochondrial NADH oxidase was assayed by measuring the decrease in the absorbance at 340 nm. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.4), 0.1 mM NADH and mitochondrial protein.[54]

NADH-chtochrome *c* reductase activity in mitochondria was determined by measuring the increase in the absorbance at 550 nm resulting from the reduction of cytochrome *c*. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 5 mM NaN_3, 0.7 mg/ml of cytochrome *c* and 0.2 mM NADH.[55]

Preparation of erythrocyte and assay for hemolysis

Blood from healthy donors was collected in heparinized tubes. Erythrocytes were separated by centrifugation from plasma and buffy coat, and were washed three times with saline. During the last washing, the cells were

centrifuged at 2,000 *g* for 10 min to obtain a constantly packed cell preparation. A 10% suspension of erythrocytes in the solution containing 152 mM NaCl and 10 mM sodium phosphate buffer (pH 7.4) was preincubated at 37°C for 5 min before addition of the same volume of 100 mM 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH) in the same buffer saline.[56] The reaction mixture was gently shaken at 37°C. At intervals during incubation, two samples were taken out from the mixture; one sample was diluted with 20 volumes of 0.15 M NaCl and the other with distilled water to yield complete hemolysis. Both samples were centrifuged at 1,000 *g* for 10 min. The absorbance of the supernatants was determined at 540 nm. The percentage of hemolysis was calculated according to the equation described by Miki et al.[36]

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