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Protection against Oxidative Damage by Dihydroflavonols in *Engelhardtia chrysolepis*

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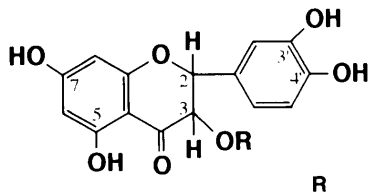
Dihydroflavonol taxifolin and its glycoside, astilbin, from *Engelhardtia chrysolepis* were evaluated as antioxidants and radical scavengers. These dihydroflavonols inhibited superoxide anion production in the xanthine/xanthine oxidase system. Microsomal lipid peroxidation induced by NADPH-cytochrome P-450 reductase was also inhibited by these flavonoids. Mitochondrial lipid peroxidation was inhibited only by the aglycon. Taxifolin protected peroxy radical-damaged mitochondria with no effect on enzyme activity. Furthermore, taxifolin and astilbin protected red cells against oxidative hemolysis. These dihydroflavonols were found to be effective for protecting subcellular systems and red blood cells against oxidative stress *in vitro*.

Key words: astilbin; taxifolin; superoxide anion; lipid peroxidation; *Engelhardtia chrysolepis*

Engelhardtia chrysolepis is a subtropical tree grown in Guangdong, Guangxi and Fujian, China. Dried materials from the heated leaves of this plant are consumed as a sweet tea to prevent obesity and are used in folk medicine as an antifebrite and anodyne. From the leaves of this plant, 3-*O*-α-L-rhamnosyl-(2*S*,3*S*)-taxifolin (neoastilbin, **3**) has been isolated as the sweet principle, together with its non-sweet isomers, astilbin (**2**), isoastilbin (**4**), and neoisoastilbin (**5**).[1,2]

Our preliminary search for antioxidative agents from tea extracts revealed that the ethanol extract of *E. chrysolepis* leaves showed potent inhibition toward lipid peroxidation. The oxidation of unsaturated fatty acids in biological membranes leads to a decrease in the membrane fluidity and a disruption of the membrane structure and function.[3,4] Cellular damage due to lipid peroxidation causes serious derangement, such as ischemia-reperfusion injury,[5] coronary arteriosclerosis,[6] and diabetes mellitus,[5] as well as being associated with aging and carcinogenesis.[7] It is well established that lipid peroxidation is one of the most deleterious reactions of oxygen free radicals in cells and tissues.[8]

The fresh leaves of *E. chrysolepis* contained 2.8–4.6% of astilbin (**2**) as a major component; other dihydroflavonol rhamnosides (**3**, **4**, and **5**) were present at concentrations of 0.2–0.5%. The ethanol extract which was preliminarily examined also contained astilbin as the main component (13.1–20.1%). This paper deals with the protective activities of dihydroflavonols in *E. chrysolepis*, and especially the aglycon, taxifolin, against oxidative damage to biological systems.



	R
1: *2R, 3R*	H
2: *2R, 3R*	Rha
3: *2S, 3S*	Rha
4: *2S, 3R*	Rha
5: *2R, 3S*	Rha

Fig. 1. Structure of Dihydroflavonols in *Engelhardtia chrysolepis*.

Materials and Methods

Chemicals. Astilbin (**2**), which had been isolated from the leaves of *E. chrysolepis*, was obtained from Maruzen Pharmaceutical Co., Ltd. Taxifolin (**1**) was obtained as a hydrolysis product of **2**. Both these compounds were identified by $[\alpha]_D$, ^{1}H- and ^{13}C-NMR spectra.

Assay of the superoxide anion. The reaction mixture consisted of a 40 mM sodium carbonate buffer (pH 10.2) containing 0.1 mM xanthine, 0.1 mM EDTA, 50 μg of protein/ml of bovine serum albumin, 25 mM nitroblue tetrazolium, and 3.3×10^{-3} units of xanthine oxidase (XOD, EC 1.2.3.2) in a final volume of 3 ml. After being incubated 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.[9]

Preparation of mitochondria and microsomes. The livers of male Wistar rats weighing 100–150 g were quickly removed and dropped into an ice-cold 3 mM Tris·HCl buffer (pH 7.4) containing 0.24 M sucrose and 0.1 mM EDTA. Mitochondria were obtained as a pellet after centrifuging at $15,000 \times g$ according to the method of Johnson and Lardy.[10] Submitochondrial particles were prepared by sonicating[11] the mitochondrial suspension for 1 min at 4 C with a Branson 450 Sonifier. Microsomes were obtained as a pellet after centrifuging at $105,000 \times g$ for 60 min,[12] and the resulting pellet then being resuspended in the buffer.

Bovine liver was homogenized in 0.25 M sucrose containing 0.1 mM EDTA (pH 7.4). The homogenate was centrifuged at $600 \times g$ for 10 min, and the supernatant was decanted and centrifuged at $15,000 \times g$ for 10 min.[10] The mitochondrial pellet was washed twice with the same solution and finally suspended in a 3 mM Tris buffer (pH 7.4) containing 0.07 M sucrose, 0.21 M mannitol and 0.1 mM EDTA.[13]

The protein concentration in each suspension was determined by the method of Lowry *et al.*[14]

Measurement of lipid peroxidation. The NADPH-dependent peroxidation of microsomal lipid was assayed by a modification of the method described by Pederson *et al.*[15] Rat liver microsomes (equivalent to 0.2 mg

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of protein) were incubated at 37°C in 1 ml of a reaction mixture containing 0.05 M Tris-HCl (pH 7.5), 2 mM ADP, 0.12 mM $Fe(NO_3)_3$, and 0.1 mM NADPH. The reaction was initiated by adding NADPH. After 5 min, 2 ml of a TCA TBA HCl reagent (15% w/v trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N HCl) and 90 μl of 2% BHT were added to the reaction mixture. The solution was then heated for 15 min in a boiling-water bath, and after cooling, the flocculent precipitate was removed by centrifuging at 1000 × *g* for 10 min. The absorbance of the thiobarbituric acid (TBA)-reactive substances was determined at 535 nm.[16]

Mitochondrial lipid peroxidation was assayed by a modification of the method described by Takayanagi *et al.*[17] Rat liver submitochondrial particles (equivalent to 0.3 mg of 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 $FeCl_3$, 10 μM rotenone, and 0.1 mM NADH. The reaction was initiated by adding NADH, and after 5 min, the reaction was terminated and lipid peroxidation was determined by the same TBA method as that used for the microsomal peroxidation.

Assay of NADH oxidase. Mitochondrial NADH oxidase was assayed by measuring the decrease in absorbance at 340 nm.[18] The reaction mixture consisted of a 50 mM phosphate buffer (pH 7.4), 0.1 mM NADH and bovine liver mitochondrial protein.

Preparation of erythrocytes and assay for hemolysis. Blood from healthy donors was collected in heparinized tubes. Erythrocytes were separated from plasma and buffy coat by centrifuging, and were washed three times with saline. During the last wash, the cells were centrifuged at 2000 × *g* for 10 min to obtain a consistently packed cell preparation. A 10% suspension of erythrocytes in a solution containing 152 mM NaCl and a 10 mM sodium phosphate buffer (pH 7.4) was preincubated at 37°C for 5 min before adding the same volume of 100 mM 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH) in the same saline buffer. The reaction mixture was then gently shaken at 37°C. At intervals during the incubation, two samples were taken from the mixture; one 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 1000 × *g* for 10 min, and the absorbance of each supernatant was determined at 540 nm. The percentage hemolysis was calculated according to the equation described by Miki *et al.*[19]

Results

Effect on generation of the superoxide anion

Oxidative enzymes such as xanthine oxidase produce the O_2^- radical as a normal product from the one-electron reduction of oxygen, resulting in tissue injury.[20] The dihydroflavonols inhibited the generation of the superoxide anion in a concentration-dependent manner as shown in Fig. 2. The 50% inhibitory concentrations of astilbin and taxifolin were 6.2 and 1.8 μg/ml, respectively.

Effect on microsomal lipid peroxidation

Membranal lipids are particularly susceptible to oxidation, not only because of their high polyunsaturated fatty acid content, but also because of their association in the cell membrane with enzymic and nonenzymic systems capable of generating free radical species.[21] Microsomes, especially the smooth-surfaced endoplasmic reticulum, easily produce lipid peroxides and are thought to supply the peroxidation products to other tissues.[22] NADPH-cytochrome P-450 reductase is involved in NADPH-induced microsomal lipid peroxidation. Lipid peroxidation, which can be measured by the TBA method, occurs when rat liver microsomes are incubated with Fe(III)-ADP/NADPH.[15] Dihydroflavonols in *E. chrysolepis* inhibited the production of lipid peroxides induced by microsomal NADPH oxidation. As shown in Fig. 3, taxifolin was more effective in preventing microsomal lipid peroxidation, almost 80% inhibition being observed at 10 μg/ml.

Effect on mitochondrial lipid peroxidation

Since redox reactions frequently occur in mitochondria, mitochondria are constantly susceptible to oxidative stress.[23] Electrons at least two sites of the mitochondrial electron transport system leak and react with oxygen to generate superoxide anions and, subsequently, hydrogen peroxide.[24] Lipid peroxides produced by the hydroxyl radical (·OH) derived from H_2O_2 affect the mitochondrial function.[25] Lipid peroxidation by submitochondrial particles is supported by NADH or NADPH in the presence of ADP and Fe(III).[17] As shown in Fig. 4, the inhibition of mitochondrial lipid peroxidation by dihydroflavonol was more moderate than that of microsomes. Taxifolin at 30 μg/ml inhibited the lipid peroxidation completely, although, astilbin had no effect at 30 μg/ml.

Effect on mitochondrial function

Many flavonoids are known to prevent lipid peroxidation.[26,27] Taxifolin has also been reported to inhibit brain mitochondrial lipid peroxidation that is induced by ascorbic acid or ferrous sulfate.[27] However, the inhibition of mitochondrial enzymes by flavonoids may contribute to their cytotoxic activity. Some flavonoids have been reported to inhibit NADH oxidase and succinate oxidase

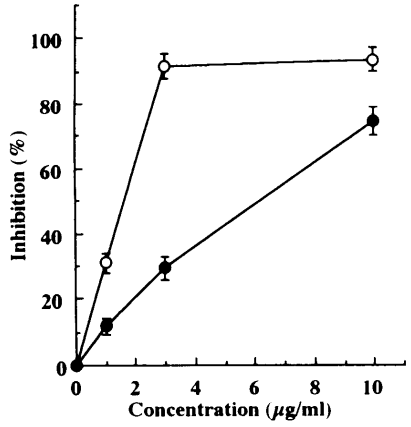

Fig. 2. Inhibitory Effects of Astilbin and Taxifolin on the Generation of the Superoxide Anion.

Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ●, astilbin; ○, taxifolin.

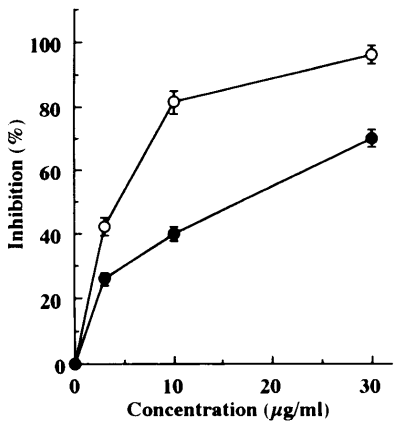

Fig. 3. Effects of Astilbin and Taxifolin on Rat Liver Microsomal Lipid Peroxidation.

Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ●, astilbin; ○, taxifolin.

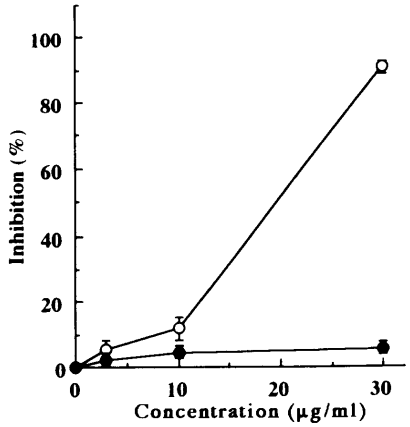

Fig. 4. Effects of Astilbin and Taxifolin on Rat Liver Mitochondrial Lipid Peroxidation.

Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ●, astilbin; ○, taxifolin.

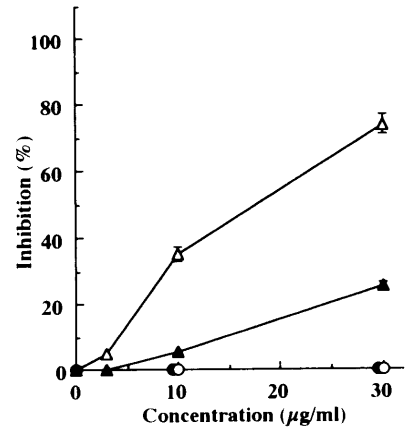

Fig. 5. Effects of Astilbin and Taxifolin on Bovine Liver Mitochondrial NADH Oxidase.

Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ●, astilbin; ○, taxifolin; ▲, quercitrin; △, quercetin.

in liver mitochondria,[28,29] or to stimulate the generation of reactive oxygen species.[29,30] As shown in Fig. 5, when the NADH oxidase activity in bovine liver mitochondria was measured, 70% and 24% inhibition values were observed with 30 μg/ml of quercetin and quercitrin (a common antioxidative flavonoid and its rhamnoside), respectively. On the other hand, taxifolin and astilbin had no effect on mitochondrial NADH oxidase at the same concentration.

Taxifolin inhibited mitochondrial lipid peroxidation without affecting mitochondrial enzyme activities. This suggests that taxifolin may be effective for preventing the functional depression of mitochondria. When mitochondria were incubated in a lipid peroxidation system, the NADH oxidase and succinate oxidase activity decreased.[31,32] Lipid peroxidation has been induced by the thermal decomposition of azo-initiator AAPH.[33] The enzyme activity of NADH oxidase in liver mitochondria decreased by almost 50% during 60 min of incubation with AAPH. As shown in Fig. 6, taxifolin at 10 μg/ml, which had no effect on mitochondrial NADH oxidase activity, protected against the AAPH-induced mitochondrial degeneration.

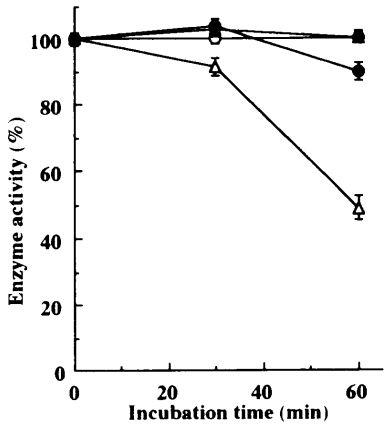

Fig. 6. Effects of Taxifolin on the Mitochondrial Respiratory Chain Treated with AAPH.

Mitochondrial respiratory activity was assayed as NADH oxidase activity. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ○, control; ▲, 10 μg/ml of taxifolin; △, 10 μg/ml of AAPH; ●, AAPH + taxifolin.

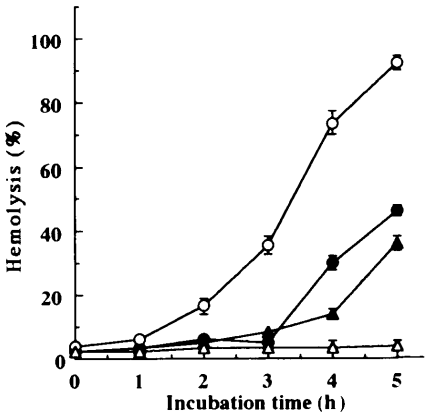

Fig. 7. Inhibition of Oxidative Hemolysis by Astilbin and Taxifolin in Human Erythrocytes.

Astilbin or taxifolin was added at a final concentration of 10 μg/ml. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ●, astilbin; ▲, taxifolin; ○, control; △, without AAPH.

Effect on oxidative hemolysis

The lipids in the red cell membrane are highly unsaturated, and the red cells are exposed to higher oxygen tension in comparison with all other tissues. Furthermore, the red cells are packed with hemoglobin, one of the most powerful catalysts capable of initiating lipid peroxidation.[34] When human erythrocytes were incubated in air at 37°C, they were stable and little hemolysis occurred within 5 hours. The peroxy radicals generated by thermal decomposition of azo-initiator AAPH have induced free radical chain oxidation in erythrocyte membranes.[19] During 4 hours of incubation with AAPH, 80% of erythrocytes were damaged. However, astilbin and taxifolin at 10 μg/ml inhibited the lysis of human red cells subjected peroxy radical attack as shown in Fig. 7.

Discussion

Some flavonoids are known to be effective inhibitors of superoxide generation by xanthine oxidase.[35] Dihydroflavonols in *E. chrysolepis* were also potent scavengers of superoxide anions, the aglycone being more effective than the rhamnoside. Quercetin, a flavonol having the same substitution as taxifolin, has also been reported to be more

effective for inhibiting superoxide generation by xanthine oxidase than its rhamnoside.[35] Taxifolin and astilbin inhibited the microsomal lipid peroxidation induced by Fe(III)-ADP/NADPH, but the rhamnoside had little effect on the mitochondrial lipid peroxidation induced by Fe(III)-ADP/NADH. This may be attributable to the difference in lipophilicity among these subcellular fractions.

Taxifolin has been reported to inhibit the brain mitochondrial lipid peroxidation induced by ascorbic acid or ferrous sulfate,[27] and the microsomal lipid peroxidation induced by carbon tetrachloride.[36] It has been reported that 3,5-dihydroxyl and 3′,4′-dihydroxyl substitutions, a 2,3-double bond and a 4-keto group, conferred antioxidative activity upon the flavonoid.[27] Quercetin, one of this type of flavonoids, is known to be an antioxidant, but has been reported to inhibit mitochondrial respiratory electron transport.[28] This confirms our present result, although, the dihydroflavonol, taxifolin, having the same substitution of hydroxyl groups as quercetin, had no effect on mitochondrial NADH oxidase up to 30 μg/ml. Furtheromore, this flavonoid maintained the enzyme activity against oxidative stress.

The present results show that the dihydroflavonols in *E. chrysolepis* were effective for protecting tissues and cells against various types of oxidative stress. Flavonoid glycosides are known to be hydrolyzed by intestinal bacteria to generate the corresponding aglycones.[37,38] Thus, astilbin may also be metabolized, and taxifolin may act as an antioxidant mainly in the case of oral administration. The effect of astilbin on lipid peroxidation *in vivo* was recently studied, and the antioxidative activity of this dihydroflavonol glycoside was suggested to be derived from taxifolin.[39]

Taxofolin has also recently been reported to decrease the serum cholesterol level in rats.[40] Astilbin and neoastilbin were clarified to inhibit lens aldose reductase[41] concerned with the formation of cataracts in diabetics.[42,43] Lipid peroxidation is also one of the causes of cataracts,[44,45] and lipid peroxides increasing in a cataractic lens[46] are prevented by antioxidants.[47] The various biological activities of *E. chrysolepis* are of considerable interest for the food industry and for preventive medicine. The antioxidative effects of these dihydroflavonols *in vivo* are now under investigation.

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