Mechanism of Antioxidant Action of Pueraria Glycoside (PG)-1 (an Isoflavonoid) and Mangiferin (a Xanthonoid)

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The antioxidant activities of pueraria glycoside (PG)-1 (isoflavonoid) and mangiferin (xanthonoid) were studied and compared with PG-3 and daidzein (isoflavonoids) and with wogonin (flavonoid). PG-1 and mangiferin rapidly scavenged 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and inhibited lipid peroxidation which was initiated enzymatically by reduced nicotinamide adenine dinucleotide phosphate (NADPH) or non-enzymatically by ascorbic acid or Fenton's reagent ($H_2O_2+Fe^{2+}$) in rat liver microsomes. Wogonin inhibited the enzymatically induced lipid peroxidation but had no scavenging effect on DPPH radical or on the non-enzymatic peroxidation. PG-3 and daidzein did not show any of these effects. Formation of Fe^{2+} by NADPH-dependent cytochrome P-450 reductase was inhibited by wogonin, but not by PG-1 or mangiferin. PG-1 and mangiferin had no effect on terminating radical chain reaction during the lipid peroxidation in the enzymatic system of microsomes or in the linoleic acid hydroperoxide-induced peroxidation system.

These results suggest that PG-1 and mangiferin have an antioxidant activity, probably due to their ability to scavenge free radicals involved in initiation of lipid peroxidation. In contrast, wogonin may affect NADPH-dependent cytochrome P-450 reductase action, since it inhibited only the enzymatically induced lipid peroxidation.

Keywords pueraria glycoside (PG)-1; mangiferin; isoflavonoid; xanthonoid; antioxidant; radical scavenger; lipid peroxidation; microsome

It is well known that flavones, which are widely distributed in the plant kingdom, have wide ranges of biochemical and pharmacological activities.[1] Many of them have potent antioxidant activities such as scavenging superoxide anion,[2] hydroxyl radical[3] and peroxy radical[4] or quenching singlet oxygen,[5] thus inhibiting lipid peroxidation in the biological system *in vitro*.[2,6] These activities are thought to afford protective effects against hepatic[7] and vascular disorders,[8] and also anti-inflammatory and anti-allergic effects.[1a] The isoflavones, structurally related to flavones, are also known to inhibit microsomal lipid peroxidation.[9] Recently isoflavones were reported to inhibit tyrosine kinase[10] and inositol phospholipid turnover,[11] and to have antifungal activity.[12] On the other hand, although xanthone derivatives widely distributed in higher plants are also reported to have some pharmacological effects, such as anti-allergic[13] and anti-platelet[14] action and central nervous system stimulating and depressing effects,[15] almost nothing is known about their antioxidant activity in the biological system.

Studies on many flavonoid derivatives have suggested that the catechol moiety in their structure may be important in exhibition of the antioxidant activity. The flavones,[3,6a] having two hydroxyl groups at 3 and 4 of B-ring, and the isoflavones,[9] having the same groups at 6 and 7 of A-ring exhibited strong potency. Since pueraria glycoside (PG)-1 and mangiferin tested in the present study have catechol moiety in their structures (Fig. 1), such as 3,4-dihydroxylated B-ring in PG-1 and 6,7-dihydroxylated structure in mangiferin, it is anticipated that these compounds may also have antioxidant activities.

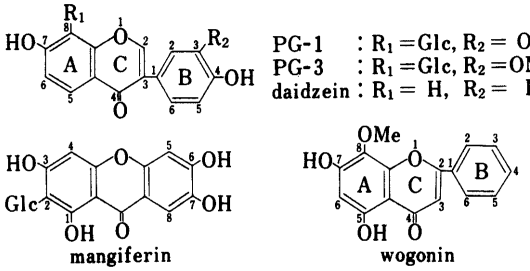

Fig. 1. Chemical Structure of the Compounds Tested

PG-1, pueraria glycoside-1; PG-3, pueraria glycoside-3.

In the present work, therefore, we studied the antioxidant effect of PG-1 and mangiferin on initiation and termination of radical chain reaction in the course of lipid peroxidation, as well as the radical scavenging capacities of these substances. We also compared the effects of two other compounds structurally related to PG-1 in view of structure–activity relationships, and of wogonin which is a flavone derivative having no hydroxyl group at B-ring (Fig. 1). On the basis of the results obtained, we attempted to postulate the mechanism of their antioxidative actions on lipid peroxidation in the biological system.

Materials and Methods

Materials *dl*-α-Tocopherol and linoleic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2-Nitroso-5-(*N*-propyl-*N*-sulfopropylamino)phenol (PSAP) was from Dojin Lab. (Kumamoto, Japan). Ascorbic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were from Nacalai Tesque Inc. (Kyoto, Japan). Adenosine diphosphate (ADP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were from Kohjin Co., Ltd. (Tokyo, Japan). PG-1 (8-β-D-glucopyranosyl-7-hydroxy-3-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one), PG-3 (8-β-D-glucopyranosyl-7-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) and daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) were extracted with methanol from the roots of *Pueraria lobata* OHWI and purified by high performance liquid chromatography (HPLC), according to the method of Ohshima *et al.*[16] Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9*H*-xanthen-9-one) was extracted with methanol from the roots of *Anemarrhena asphodeloides* BUNGE and purified by HPLC.[17] Wogonin (5,7-dihydroxy-8-methoxy-2-phenyl-4*H*-1-benzopyran-4-one) was extracted with methanol from *Scutellaria baicalensis* GEORGI and also purified by HPLC.[18] Other reagents were obtained from commercial sources.

Decolorization of DPPH Each compound tested in dimethyl sulfoxide (DMSO) was added at a final concentration of 50 μM to 0.1 mM DPPH

solution in ethanol, and continuous monitoring of the change with time in absorbance at 517 nm was started immediately.[19]

Preparation of Rat Liver Microsomes Rat liver obtained from male Wistar rats 7—8 weeks of age was homogenized in a medium composed of 1.15% KCl, 0.2% nicotinamide, 50 mM mannitol and 50 mM Tris, pH 7.5. The supernatant after centrifugation of the homogenate at $1200 \times g$ for 10 min at 4 °C, was centrifuged at $11000 \times g$ for 20 min. This supernatant was further centrifuged at $100000 \times g$ for 30 min at 4 °C, and the pellets obtained were resuspended in the same medium and stored at −80 °C until use. Protein concentration was determined by the method of Lowry *et al.*[20]

Lipid Peroxidation of Microsomes The microsomal suspension (0.5 mg protein/ml) had been preincubated with 3 mM ADP and 0.15 mM $FeCl_3$ at 37 °C for 10 min before each compound to be tested was added. After a further 10 min incubation, lipid peroxidation was initiated enzymatically by adding 0.5 mM NADPH or non-enzymatically by 0.1 mM ascorbic acid. In Fenton reaction,[21] microsomal suspension (0.5 mg protein/ml) was preincubated with 3 mM ADP and treated with each compound as above, and then 1 mM H_2O_2 and 0.15 mM $FeSO_4$ were added. After incubation for an appropriate time, the lipid peroxidation was assayed by the thiobarbituric acid (TBA) method[22] and the extent of peroxidation was expressed as the change in absorbance of thiobarbituric acid-reactive substances (TBA-RS) at 535 nm. The amount of TBA-RS which existed in the mixture before starting the peroxidation reaction was subtracted from each value.

Radical Chain Reaction by Linoleic Acid Hydroperoxide Linoleic acid (1 mmol) was left in the dark under air at 50 °C for 72 h to obtain linoleic acid hydroperoxide. After the reaction, this preparation contained 51—55 μmol of linoleic acid hydroperoxide, and was used without further purification as the source of the hydroperoxide. To the mixture of 10 mM fresh linoleic acid dispersed in phosphate buffered saline (140 mM NaCl, 10 mM phosphate, pH 7.4) containing 50 μM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM $FeSO_4$, the preparation comprising linoleic acid hydroperoxide obtained as above was added to make a final hydroperoxide concentration of 16 μM, and radical chain reaction was started at 37 °C. After an appropriate time the lipid peroxides were determined by TBA method.

Determination of Ferrous Ion Lipid peroxidation in microsome was initiated enzymatically by the addition of NADPH and $FeCl_3$ as described above. After an appropriate period of incubation at 37 °C, an aliquot of the reaction mixture was mixed with 10 mg/ml PSAP and incubated at 37 °C for 1 min. The concentration of Fe^{2+} was calculated from the absorbance of Fe^{2+}-PSAP complex formed at 756 nm using molecular extinction coefficient of PSAP (45000).[23]

Results

Effect on Decolorization of DPPH The radical scavenging capacities of five compounds were examined and compared with that of *dl*-α-tocopherol, by determining the decrease in optical density of DPPH, a stable free radical. As shown in Fig. 2, PG-1 and mangiferin caused rapid decolorization of DPPH solution, indicating their marked radical scavenging potencies which were as strong as *dl*-α-tocopherol, whereas wogonin, daidzein and PG-3 had almost no effect.

Antioxidative Effect on Lipid Peroxidation in Microsomes We examined antioxidant capacity of each compound in the biological lipid peroxidation system using rat liver microsomes. Addition of NADPH and Fe^{3+} to microsomal suspension increased TBA-RS time-dependently up to 60 min, as shown in Fig. 3. When microsomes had been pretreated with each compound tested, the production of TBA-RS was remarkably inhibited by PG-1, mangiferin, wogonin and *dl*-α-tocopherol, but not by PG-3 or daidzein even at a concentration of 200 μM. Mangiferin and wogonin as well as *dl*-α-tocopherol completely inhibited it during 60 min incubation.

Since NADPH and Fe^{3+}-induced lipid peroxidation is triggered by activation of NADPH-dependent cytochrome P-450 reductase, the antioxidant activity of the compounds may possibly involve their inhibitory effect on the enzyme action; hence their true antioxidant capacity such as scavenging active oxygen radical cannot be evaluated in such an enzymatic system. Therefore, we also studied

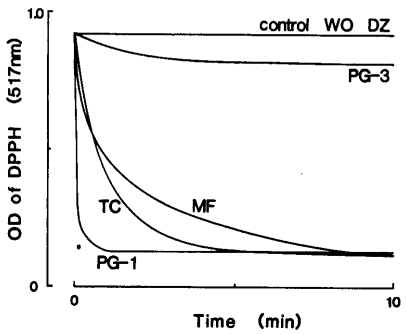

Fig. 2. Time-Dependent Decolorization of DPPH by the Action of the Compounds Tested

Each compound was added at a final concentration of 50 μM to 0.1 mM DPPH solution and the change in absorbance was monitored. Control, without any compound; PG-1, pueraria glycoside-1; PG-3, pueraria glycoside-3; DZ, daidzein; MF, mangiferin; WO, wogonin; TC, *dl*-α-tocopherol.

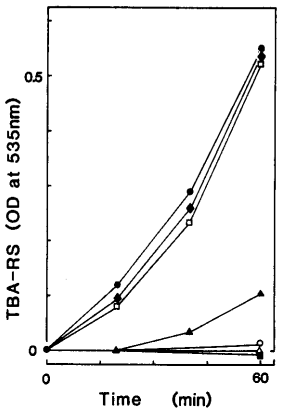

Fig. 3. Inhibitory Effect of the Compounds Tested on the Lipid Peroxidation Initiated by NADPH and $FeCl_3$ in Rat Liver Microsomes

For experimental details see Materials and Methods. The amount of peroxidated products is expressed as the change in absorbance of thiobarbituric acid-reactive substances (TBA-RS) at 535 nm. ●, control; ■, mangiferin (50 μM); ▲, PG-1 (50 μM); □, PG-3 (200 μM); ◆, daidzein (200 μM); ○, wogonin (50 μM); △, *dl*-α-tocopherol (50 μM). Each point represents the mean of three separate experiments.

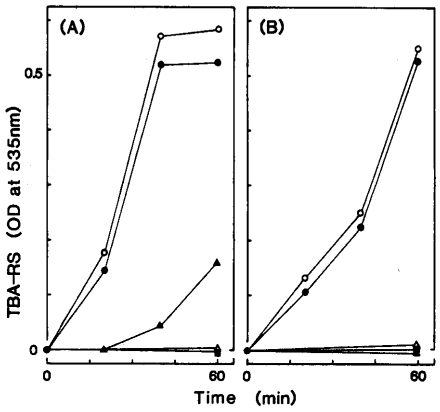

Fig. 4. Inhibitory Effect of the Compounds Tested on Lipid Peroxidation Initiated by Ascorbic Acid and $FeCl_3$ (A), or Fenton Reaction (B, $H_2O_2 + FeSO_4$) in Rat Liver Microsomes

The symbols and the concentrations used for each compound are the same as those in Fig. 3. Each point represents the mean of three separate experiments.

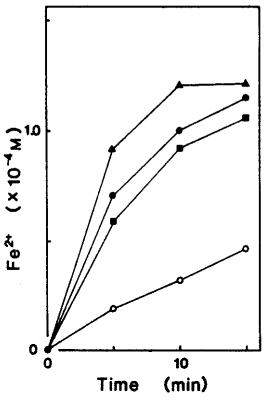

Fig. 5. Effect of the Compounds Tested on the Production of Ferrous Ion That Is Generated by NADPH-Dependent Cytochrome P-450 Reductase in Rat Liver Microsomes

For experimental details see Materials and Methods. The symbols and the concentrations used for each compound are the same as those in Fig. 3. Each point represents the mean of three separate experiments.

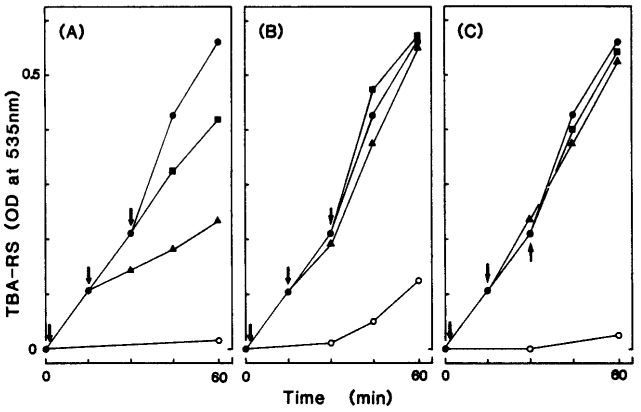

Fig. 6. Effect of the Compounds Tested on Terminating Chain Reaction of Lipid Peroxidation in Rat Liver Microsomes

Lipid peroxidation was initiated enzymatically by addition of NADPH and $FeCl_3$ to rat liver microsomes, as described in Materials and Methods. At the time 0 (○), 15 (▲) and 30 min (■) of incubation, *dl*-α-tocopherol (A), PG-1 (B) or mangiferin (C) was added to make a final concentration of 50 μM (indicated by an arrow in the figure), and TBA-RS produced was determined. ●, without addition of any compound. Each point represents the mean of three separate experiments.

the effect of these compounds on the lipid peroxidation induced non-enzymatically. As indicated in Fig. 4A, TBA-RS production induced by addition of ascorbic acid and Fe^{3+} which means circumventing cytochrome P-450 reductase action, was inhibited remarkably by mangiferin and *dl*-α-tocopherol, and rather weakly by PG-1, while it was not inhibited at all by wogonin. Similar results were obtained in TBA-RS production initiated by Fenton's reagent ($H_2O_2+Fe^{2+}$) (Fig. 4B). PG-3 and daidzein also did not show any effect (data not shown). These results suggest that PG-1 and mangiferin have an antioxidant activity to inhibit the process involving initiation and/or termination of chain reaction, and that wogonin has no such effect.

Effect on NADPH-Dependent Cytochrome P-450 Reductase Activity As demonstrated in Figs. 3 and 4, wogonin inhibited enzymatic lipid peroxidation but not the non-enzymatic one, suggesting its suppressive effect on cytochrome P-450 reductase action. Considering the fact that the initial step of enzymatic lipid peroxidation in microsome is the reduction of Fe^{3+} by the enzyme, we tried to confirm the effect of wogonin on Fe^{2+} formation

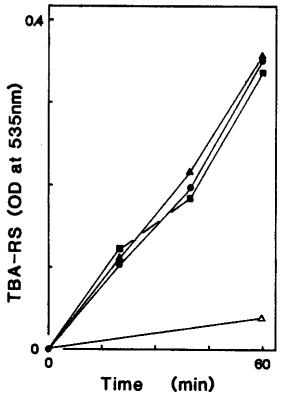

Fig. 7. Effect of the Compounds Tested on Linoleic Acid Hydroperoxide-Induced Lipid Peroxidation

Lipid peroxidation was initiated by adding autoxidated linoleic acid to fresh linoleic acid and $FeSO_4$ in the absence (●) or presence of 200 μM of PG-1 (▲) or mangiferin (■), or 50 μM of *dl*-α-tocopherol (△), as described in Materials and Methods. Each point represents the mean of three separate experiments.

as an indicator of its action on the enzyme. As seen in Fig. 5, when NADPH and Fe^{3+} were added to microsomes, the production of Fe^{2+} increased time-dependently. Wogonin inhibited this increase by about 70% after 15 min incubation, whereas PG-1 and mangiferin showed no effect. This result suggests that the inhibition of enzymatically produced TBA-RS by wogonin might be due to suppression of the enzyme action.

Effect on Termination of Radical Chain Reaction Since it is possible that the antioxidant activities of PG-1 and mangiferin are partly due to termination of the radical chain reaction during lipid peroxidation, in addition to their action to scavenge radical involved in the step of initiation, we studied such effect in the following two experiments. When PG-1 or mangiferin was added 15 and 30 min after starting enzymatic lipid peroxidation in microsomes with NADPH and Fe^{3+}, subsequent increase in TBA-RS was observed in the same extent as in the control without the added compound, while *dl*-α-tocopherol under the same condition significantly retarded the subsequent increase (Fig. 6). With higher concentration of PG-1 and mangiferin (200 μM), the same results were observed (data not shown). Furthermore, PG-1 and mangiferin had negligible effect on the formation of TBA-RS initiated by autoxidated linoleic acid and Fe^{2+} in fresh linoleic acid suspension, as shown in Fig. 7, whereas *dl*-α-tocopherol markedly inhibited the formation. These suggest that PG-1 and mangiferin do not have antioxidant ability to scavenge peroxy radical involved in lipid peroxidation.

Discussion

The NADPH-dependent lipid peroxidation in microsome is supposed to be initiated as follows[24]: (A) ADP-Fe^{3+} → ADP-Fe^{2+}, (B) ADP-$Fe^{2+}+O_2$ → ADP-Fe^{2+}-O_2, (C) ADP-Fe^{2+}-O_2 ↔ ADP-Fe^{3+}-O_2^-, (D) ADP-Fe^{3+}-$O_2^-+LH+H^+$ → ADP-$Fe^{3+}+L\cdot+H_2O_2$. The reduction of Fe^{3+} to Fe^{2+} (A) which triggers the peroxidation system, is induced enzymatically by NADPH-dependent activation of cytochrome P-450 reductase or non-enzymatically by addition of a reducing agent such as ascorbic acid. In the present work, we studied the antioxidative effect of PG-1 and mangiferin on initiation and termination of radical

chain reaction during lipid peroxidation induced by various initiators, in comparison with PG-3 and daidzein, structurally related compounds to PG-1, and with wogonin, a flavone derivative.

PG-1 and mangiferin had a strong antioxidative potency as indicated by the following observations: scavenging effect on DPPH radical (Fig. 2) and inhibition of lipid peroxidation in microsomes initiated by enzymatic (NADPH) or non-enzymatic (ascorbic acid or Fenton reaction) systems (Figs. 3 and 4). Furthermore, they did not inhibit NADPH-dependent cytochrome P-450 reductase activity, since they had no effect on the reduction of Fe^{3+} by the enzyme (Fig. 5). However, they could not exhibit antioxidant ability to terminate radical chain reaction during the lipid peroxidation in the enzymatic system of microsomes (Fig. 6) or in linoleic acid peroxide-induced system (Fig. 7). These results indicate that they have little potency on terminating chain reaction by donating a hydrogen atom to peroxy radical, as suggested by Torel *et al.*[4] Thus, we suggest that the antioxidant activity of PG-1 and mangiferin might be due to their ability to scavenge free radical associated with the initiation of lipid peroxidation.

On the other hand, wogonin inhibited the lipid peroxidation induced by NADPH and Fe^{3+} (Fig. 3), but not by ascorbic acid or Fenton reaction in microsomes (Fig. 4). Furthermore, it could not scavenge DPPH radical (Fig. 2) but did exert significant effect on NADPH-induced enzymatic reduction of Fe^{3+} (Fig. 5). These results suggest that the inhibition of NADPH-induced lipid peroxidation by wogonin might be secondary to the suppression of cytochrome P-450 reductase reaction. This inhibition may be due to its direct inhibition of the enzyme or to its membrane-stabilizing effect.

As to the structure–activity relationships for antioxidant activity of isoflavone derivatives, it has been reported by Jha *et al.*[9] that the compounds having two hydroxyl groups at the 6 and 7 positions of A-ring generally have a strong potency. In the present results, PG-1 with two hydroxyl groups at 3 and 4 of B-ring exhibited strong antioxidant activity, whereas PG-3 which possesses 3-OCH_3 group at B-ring and daidzein which lacks 3-OH group did not exert any antioxidant effect. These results indicate that the catecholic moiety of B-ring of isoflavone is important for exhibiting antioxidant potency, as reported with flavones.[3,6a] Similarly, mangiferin is thought to exhibit its antioxidant activity by the catecholic structure of two hydroxyl groups at 6 and 7 positions. In contrast, wogonin having no hydroxyl group at aromatic B-ring is considered to lack the effect on lipid peroxidation.

In conclusion, we showed that PG-1 and mangiferin have a strong antioxidant activity in the biological peroxidation system, and that their capacities might result from their action of scavenging free radicals, *e.g.*, ·OH and O_2^-, associated with initiation of lipid peroxidation, rather than terminating radical chain reaction in lipid peroxidation.

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