[Chem. Pharm. Bull. 34(3)1223—1227(1986)]

Effects of Flavonoids and Related Compounds from Mulberry Tree on Arachidonate Metabolism in Rat Platelet Homogenates

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(Received August 23, 1985)

The effects of various flavonoids and related compounds isolated from the root bark of mulberry tree on rat platelet lipoxygenase and cyclooxygenase products formed from [1-^{14}C] arachidonic acid were studied. Morusin was found to inhibit the formations of 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and thromboxane B_2 (cyclooxygenase products) more strongly than the formation of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) (12-lipoxygenase product). Oxydihydromorusin and kuwanon C were also found to inhibit the formation of thromboxane B_2 more strongly than the formations of HHT and 12-HETE. Mulberrofuran A inhibited the formations of HHT and thromboxane B_2, but it increased the formation of 12-HETE. Albanol B and mulberrofuran F did not affect arachidonate metabolism in rat platelet homogenates.

Keywords——flavonoid; mulberry tree; thromboxane B_2; rat platelet homogenate; arachidonate metabolism

Mulberry trees have been widely cultivated in China and Japan, and the leaves are used to feed silkworms. On the other hand, the root bark of the mulberry tree (*Morus alba* L. and other plants of the genus *Morus*) has been used as an anti-phlogistic, diuretic, expectorant, and laxative in Chinese traditional medicine. In a pharmacological study, the extract of the root bark was reported to show a marked hypotensive effect.[1] Nomura *et al.*[2] have isolated many phenolic constituents from this plant.

Platelet cyclooxygenase is known to catalyze the initial reaction that leads to the formation of prostaglandin H_2(PGH_2), which is converted to thromboxane A_2(TXA_2) by thromboxane synthetase and to other eicosanoids such as PGD_2 and PGE_2.[3] TXA_2 is readily transformed to TXB_2, which is a stable form. TXA_2 is known to be a potent leukocyte chemotactic substance[4] and a potent platelet aggregator.[5] A number of non-steroidal anti-inflammatory drugs (*e.g.*, aspirin and indomethacin) have been found to inhibit the formation of cyclooxygenase products such as 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), TXB_2 and prostaglandins, but do not inhibit the lipoxygenase enzyme.[6,7]

In the present work, we examined the effects of various flavonoids and related compounds isolated from the root bark of mulberry tree on arachidonate metabolism in rat platelet homogenates.

Materials and Methods

Materials——Rat blood was obtained from normally fed Wistar–King strain rats (300—400 g). Washed platelets were prepared by differential centrifugation. [1-^{14}C] Arachidonic acid was purchased from Amersham Co. Morusin, oxydihydromorusin, kuwanon C, mulberrofuran A, mulberrofuran F and albanol B were isolated from the root bark of mulberry tree as described by Nomura *et al.*[2] The chemical structures of these compounds are shown in Fig. 1.

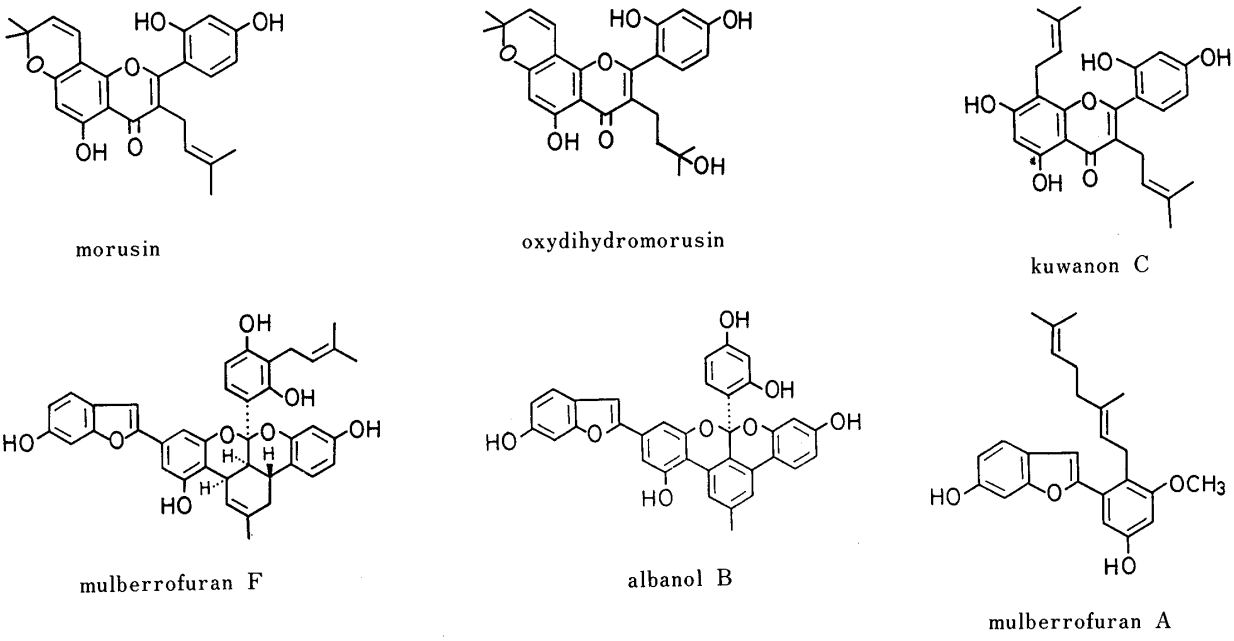

Fig. 1. Structures of Various Flavonoids and Related Compounds

Precoated Silica gel 60 TLC plastic sheets were obtained from Merck Co. Other chemicals were of reagent grade.

Measurements of the [1-^{14}C] Arachidonic Acid Cascade in Homogenates of Rat Platelets——Sonication of rat platelets was performed using a Sonifier Cell Disruptor (Branson Sonic Power, Co.). An ethanol solution of [1-^{14}C] arachidonic acid (10 μCi/ml) was preserved at -40 °C, and then 0.1 ml of the solution was diluted by the addition of 0.9 ml of Hepes/saline buffer (pH 7.4) and used for this study (1 μCi/ml). Test compounds were suspended in Hepes/saline buffer (pH 7.4) by using the sonicator. Sonicated platelets (5 mg protein/ml) (130 μl) were preincubated with test compounds (20 μl) for 5 min at 37 °C. Then, [1-^{14}C] arachidonic acid (50 μl, 0.05 μCi/tube) was added to give a final concentration of 0.84 nmol/0.2 ml tube and the mixture was incubated for 5 min at 37 °C. The reaction was stopped by adding 0.5 N formic acid (200 μl) and the products were extracted with 8 volumes of EtOAc. The EtOAc phase was evaporated under N_2 gas. The residue was dissolved in a small amount of EtOAc (40 μl), applied to precoated Silica gel 60 TLC plastic sheets, and developed with EtOAc–2,2,4-trimethylpentane–acetic acid–water (100 : 50 : 20 : 100, v/v, upper phase) or $CHCl_3$–MeOH–acetic acid–water (135 : 12 : 1.5 : 1.2, v/v). There was no effect of the organic solvent at the concentrations used on the response of arachidonate metabolism in rat platelet homogenates. These metabolites were identified by comparison with authentic compounds and by gas chromatography-mass spectrometry as described previously.[8] Radioactive spots were detected by autoradiography, cut out with scissors and counted in a liquid scintillation counter. Protein was determined by the method of Lowry *et al.*[9] with bovine serum albumin as a standard.

Results

When arachidonic acid was incubated with sonicated rat platelet homogenates, it was converted to three major compounds, 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), HHT and thromboxane B_2. The radioactivities of 12-HETE (12-lipoxygenase product), HHT and thromboxane B_2 (cyclooxygenase products) formed in the control were 34.5 ± 1.43, 15.8 ± 1.04 and 13.1 ± 1.01 ($\times 10^3$ cpm) (means $\pm$ standard errors for 18 experiments), respectively. The amounts of 12-HETE, HHT and thromboxane B_2 formed after a 5 min incubation were found to be proportional to the amount of homogenate present (data not shown).

Figures 2(a)—(e) show the effects of morusin, oxydihydromorusin, kuwanon C, mulberrofuran A and indomethacin on the formations of 12-HETE, HHT and thromboxane B_2. As shown in Fig. 2(a), the formations of HHT and thromboxane B_2 were inhibited by morusin dose-depenedently, while the formation of 12-HETE was slightly stimulated at low concentrations (10^{-6}—10^{-5} M) but was inhibited at high concentrations (10^{-4}—10^{-3} M).

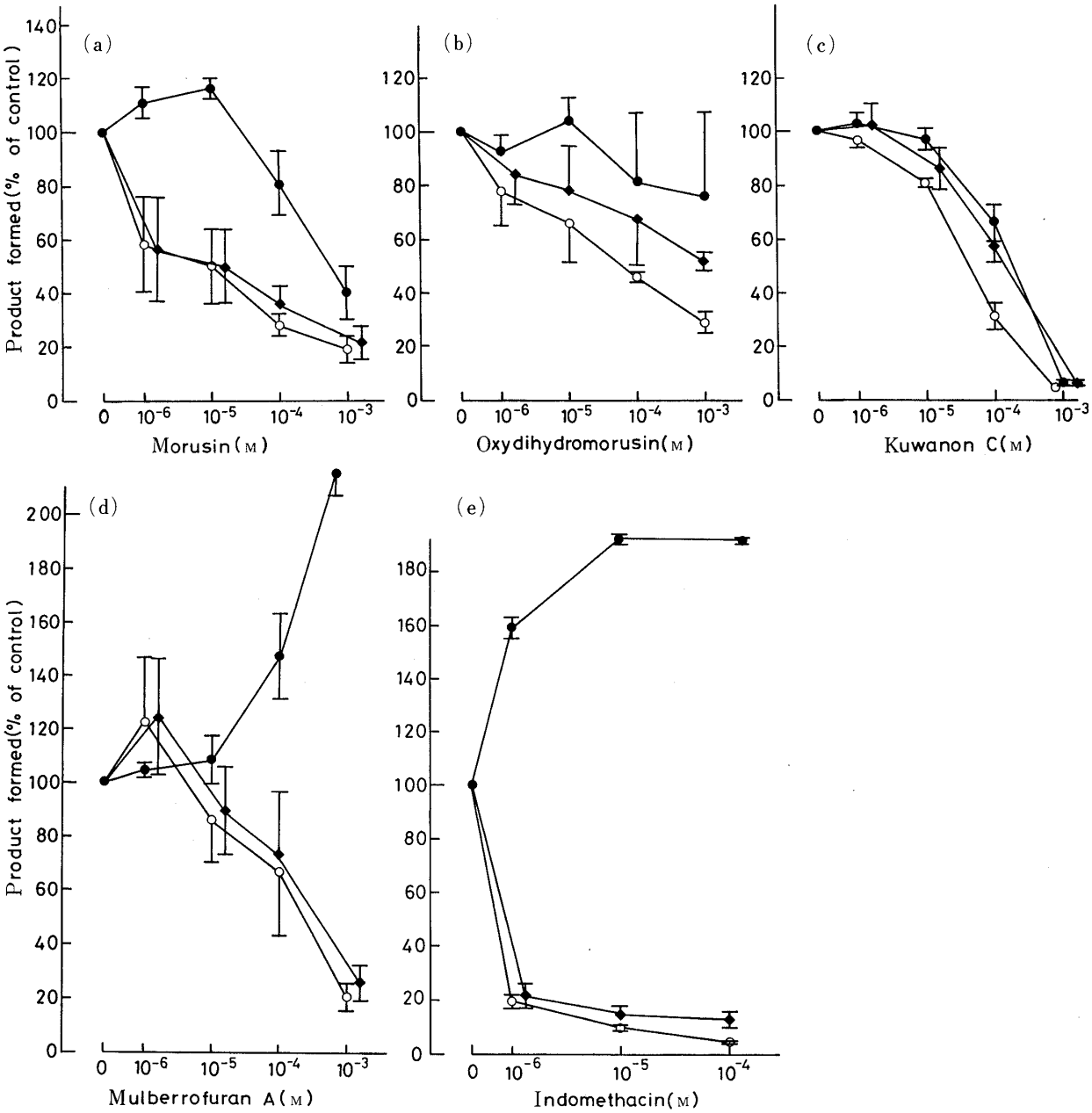

Fig. 2. Effects of Morusin (a), Oxydihydromorusin (b), Kuwanon C (c), Mulberrofuran A (d) and Indomethacin (e) on Arachidonate Metabolism in Rat Platelet Homogenates

Platelets, (5 mg protein/ml) (130 μl) sonicated in 25 mM Hepes/125 mM NaCl buffer (pH 7.4) containing 2 mM EDTA were preincubated with various flavonoids and related compounds (20 μl) for 5 min at 37 °C. After addition of [1-^{14}C] arachidonic acid (50 μl, 0.05 μCi/tube), the mixture was incubated for 5 min at 37 °C. The reaction was stopped by adding 0.5 N formic acid (200 μl). The products were extracted with ethyl acetate, and chromatographed on a silica gel TLC plastic sheet in ethyl acetate–2,2,4-trimethylpentane–acetic acid–water (100 : 50 : 20 : 100, v/v, upper phase). Radioactivities in the arachidonic acid, 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), 12-hydroxy-5,8,10-hetadecatrienoic acid (HHT) and thromboxane B_2 fractions counted by liquid scintillation spectrometry.

Values are the means ± standard errors for 3 experiments. ●, 12-HETE; ◆, HHT; ○, thromboxane B_2.

Oxydihydromorusin also inhibited the formations of HHT and thromboxane B_2 dose-dependently, while it showed no effect on the formation of 12-HETE. (Fig. 2(b)). Furthermore, oxydihydromorusin inhibited the formation of thromboxane B_2 more strongly than the

formation of HHT. As shown in Fig. 2(c), the formations of 12-HETE, HHT and thromboxane B_2 were inhibited by kuwanon C in a dose-dependent manner. On the other hand, mulberrofuran A also inhibited the formations of HHT and thromboxane B_2, though less strongly, while it stimulated the formation of 12-HETE from arachidonic acid in platelet homogenates at concentrations of 10^{-6}—10^{-3} M. Mulberrofuran F and albanol B did not affect arachidonate metabolism in rat platelet homogenates (data not shown). An anti-inflammatory drug, indomethacin, also inhibited the formation of HHT and thromboxane B_2 dose-depenedently, while it stimulated the formation of 12-HETE (Fig. 2(e)).

Discussion

The present investigation has demonstrated that various phenolic compounds isolated from the root bark of the cultivated mulberry tree significantly affect arachidonate metabolism in rat platelet homogenates. A number of non-steroidal anti-inflammatory drugs such as aspirin and indomethacin have been shown to inhibit the formation of cyclooxygenase products, but lipoxygenate enzymes are not inhibited.[6,7] Among the compounds used in this study, morusin and kuwanon C inhibited both cyclooxygenase and 12-lipoxygenase at high concentrations (10^{-4}—10^{-3} M), but oxydihydromorusin selectively inhibited the formation of the cyclooxygenase product, thromboxane B_2 without affecting the formation of 12-HETE (12-lipoxygenase product). These findings suggest that a 2,2-dimethylchromene ring of angular type in the A-ring of the flavone and a free phenolic hydroxyl group at the C-5 position in the flavone skeleton may be essential for selective inhibition of the formation of thromboxane B_2. Generally, the inhibitory effects of prenylflavones such as morusin, oxydihydromorusin and kuwanon C on the formations of HHT and thromboxane B_2 were stronger than those of 2-arylbenzofuran derivatives such as mulberrofuran A, mulberrofuran F and albanol B.

In terms of structure–activity relationship, two elements appear to be important. Both the 2,2-dimethylchromene ring of angular type in the A-ring and the γ,γ-dimethylallyl group at the C-3 position in the γ-pyrone ring of the flavone skeleton might be essential for the inhibition of the formation of cyclooxygenase products, HHT and thromboxane B_2. In the previous paper,[10] we reported that a number of flavonoids, (2*S*),2′,5,6′,7-tetrahydroxyflavanone, (2*R*,3*R*),2′,3,5,6′,7-pentahydroxyflavanone, 2′,5,5′,7-tetrahydroxy-6′,8-dimethoxyflavone, wogonin (5,7-dihydroxy-8-methoxyflavone) and skullcapflavone II (2′,5-dihydroxy-6,6′,7,8-tetramethoxyflavone)isolated from Scutellariae Radix inhibited the formation of the cylcooxygenase product, HHT, in leukocyte homogenate. Therefore, a free phenolic hydroxyl group at C-5 in the A-ring and a free phenolic hydroxyl group at the C-2′ position in the B-ring of the flavone skeleton might be required for the inhibition of the formation of the cyclooxygenase products, HHT and thromboxane B_2. Among 2-arylbenzofuran derivatives, mulberrofuran A only inhibited the formations of HHT and thromboxane B_2, while the other 2-arylbenzofuran derivatives, mulberrofuran F and albanol B had no effect. These results suggest that the geranyl group having a two double bond system at the C-2′ position in the benzene ring may be essential for the inhibition of the formations of thromboxane B_2 and HHT.

In this study, it was found that the inhibitory effects of indomethacin on the formations of HHT and thromboxane B_2 were stronger than those of various flavonoids and related compounds isolated from the root bark of mulberry tree.

Thromboxane A_2 is known to be involved in various inflammatory processes, such as formation of leukocyte chemotactic substance[4] and platelet aggregation.[5] The anti-inflammatory action of the root bark of the cultivated mulberry tree may be due to the inhibition of formation of the cyclooxygenase product, thromboxane B_2, by various phenolic

compounds.

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