Journal of Natural Products
Vol. 49, No. 4, pp. 639-644, Jul-Aug 1986

EFFECTS OF PHENOLIC CONSTITUENTS FROM THE MULBERRY TREE ON ARACHIDONATE METABOLISM IN RAT PLATELETS

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ABSTRACT.—The effects of various phenolic compounds isolated from the rootbark of the mulberry tree on rat platelet cyclooxygenase and lipoxygenase products formed from (1-^{14}C)arachiodonic acid were studied. Kuwanons G and H, sanggenon C, and mulberrofuran Q at concentrations of 10^{-3} to 10^{-4}M inhibited the formation of 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and thromboxane B_2 (cyclooxygenase products); however, they increased the formation of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) (a lipoxygenase product). Sanggenon D and mulberrofuran J at concentration of 10^{-3}M inhibited the formation of HHT, thromboxane B_2, and 12-HETE. Mulberrofuran G at a concentration of 10^{-3}M inhibited the formation of HHT, thromboxane B_2, and 12-HETE, while at 10^{-5}M, it inhibited the formation of 12-HETE without affecting the formations of HHT and thromboxane B_2. Kuwanon M and mulberroside A did not affect arachidonate metabolism in rat platelets.

Mulberry trees are cultivated in China and Japan, and their leaves are used to feed silkworms, while the rootbark of the mulberry tree (*Morus alba* L. and other plants of the genus *Morus*) is a traditional medicine used as an antipholgistic, diuretic, expectorant, and laxative. Moreover, extracts of the rootbark show a marked hypotensive effect (1), and Nomura *et al.* (2-13) isolated some phenolic constituents with significant hypotensive effects from this plant.

Previously, we reported that prenylflavones such as morusin and kuwanon C inhibited the formation of HHT and thromboxane B_2 from (1-^{14}C)arachidonic acid in rat platelet homogenates (14).

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 by thromboxane synthetase and to other eicosanoids such as PGD_2, PGE_2, and $PGF_{2\alpha}$ (15). Thromboxane A_2 is readily transformed to thromboxane B_2, which is a stable form. Thromboxane A_2 is known to be a potent leukocyte chemotactic substance (16) as well as a potent platelet aggregator (17). A number of nonsteroidal anti-inflammatory drugs (e.g., aspirin and indomethacin) have been found to inhibit the formation of cyclooxygenase products such as HHT, thromboxane B_2, and prostaglandins but not to inhibit the lipoxygenase (18,19). On the other hand, it is known that platelet lipoxygenase catalyzes the initial reaction that leads to formation of 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) (18-20). This 12-HPETE is readily transformed to the stable form, 12-HETE. Nakao *et al.* (21) reported that 12-HETE at a very low concentration ($6\times10^{-15}-6\times10^{-13}$ g/ml) significantly stimulated aortic smooth muscle cell migration, and that the locomotion induced by 12-HETE was chemokinetic. From the above results, Nakao *et al.* (21) suggested the physiological importance of the platelet 12-lipoxygenase product of arachidonic acid in the early phase of atherosclerosis. In contrast, Takenaga *et al.* (22) recently found that 12-HPETE caused dose-dependent inhibition of collagen-induced platelet aggregation.

Furthermore, they observed that 12-HPETE inhibited arachidonic acid release from platelet membrane phospholipids.

In the present work, we examined the effects of various phenolic compounds isolated from the rootbark of the mulberry tree on arachidonate metabolism in rat platelets.

MATERIALS AND METHODS

MATERIALS.—Rat blood was obtained from normally fed Wistar-King strain rats (300-400 g), and

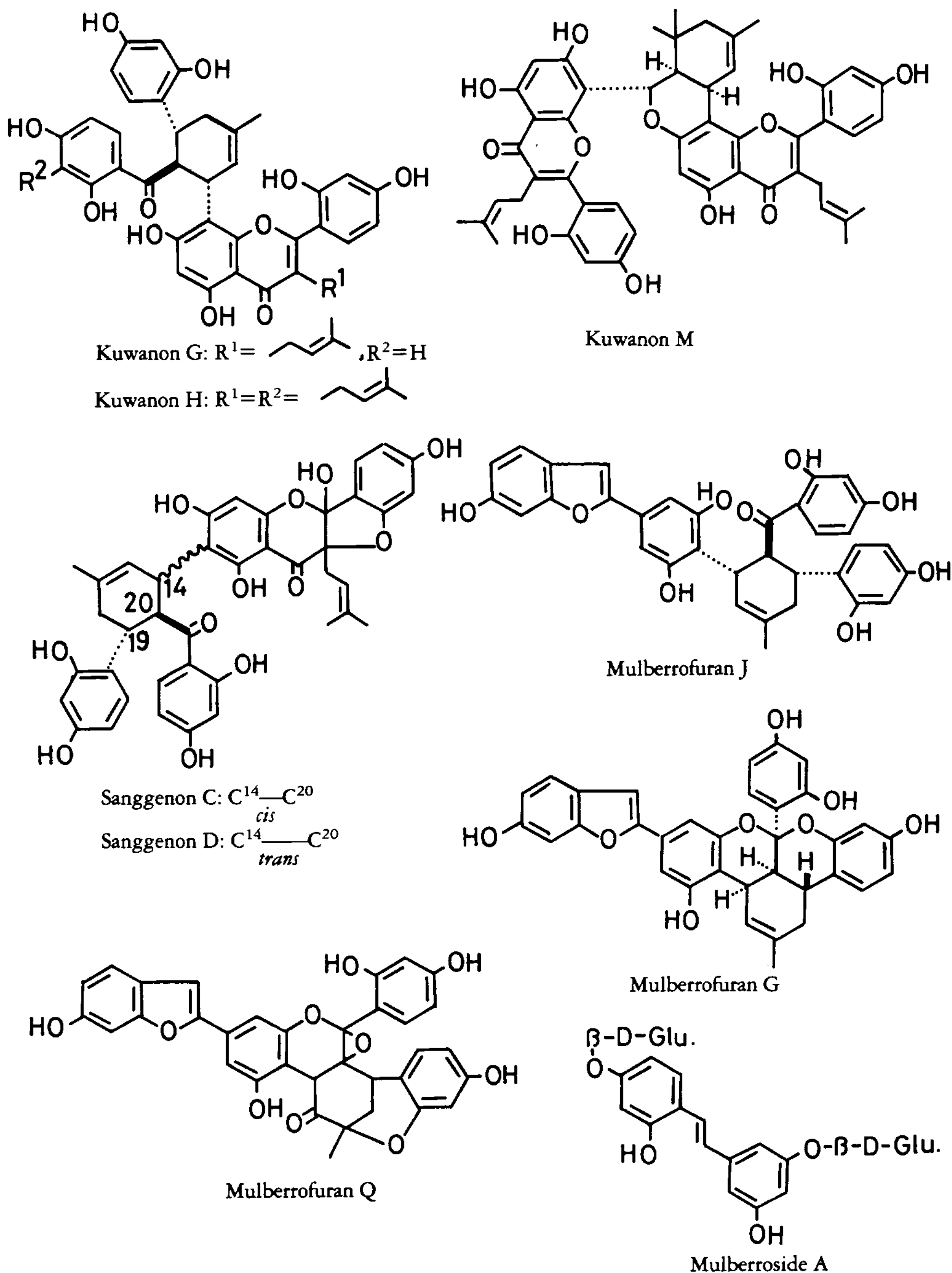

Figure 1. Chemical structures of test compounds.

washed platelets were prepared from the blood by differential centrifugation. From Amersham Co. we purchased (1-^{14}C)arachidonic acid, which we stored as an ethanolic solution (10 μCi/ml) at −40°. Before use, 0.1 ml of the solution was diluted with 0.9 ml of 25 mM Hepes/125 mM NaCl buffer (pH 7.4) to give for this study 1 μCi/ml. Kuwanons G, H, and M; sanggenons C and D; mulberrofurans J, G, and Q; and mulberroside A were isolated from the rootbark of the mulberry tree as described by Nomura *et al.* (2-13). Test compounds (10^{-2}M) were dissolved in EtOH-propylene glycol (3:1). Organic solvents, except propylene glycol, were then evaporated completely under a N_2 gas stream, and to them Hepes/saline buffer (pH 7.4) was added. Propylene glycol was used because it helps water-insoluble drugs dissolve into aqueous buffer. The high concentrations (10^{-3}M) of test compounds were nearly dissolved in the platelet-rich medium. At concentrations of 10^{-4}-10^{-6}M, test compounds were completely dissolved in the reaction medium. The chemical structures of these compounds are shown in Figure 1. Precoated silica gel tlc plastic sheets were obtained from Merck Co. Other chemicals were reagent grade.

MEASUREMENTS OF THE (1-^{14}C)ARACHIDONIC ACID CASCADE IN RAT PLATELETS.—Rat platelets (6×10^5 cells/μl) (130 μl) were preincubated with test compounds (20 μl) for 5 min at 37°. Then, (1-^{14}C)arachidonic acid (50 μl, 0.05 μCi/tube) was added to give a final concentration of 0.84 nmol at a final volume of 200 μl, and the mixture was incubated for 5 min at 37°. The reaction was stopped by adding 0.5 N HCOOH (200 μl), and the products were extracted with 8 volumes of EtOAc. The EtOAc phase was evaporated under N_2. The residue was dissolved in a small amount of EtOAc (40 μl), applied to precoated silica gel 60 Tlc plastic sheet, and developed with two organic solvents: (a) EtOAc-2,2,4-trimethylpentane-HOAc-H_2O (100:50:20:100, v/v, upper phase) and (b) $CHCl_3$-MeOH-HOAc-H_2O (13:12:1.5:1.2, v/v). These metabolites were identified by comparison with authentic samples and by gc/ms as described previously (23,24). Radioactive spots were detected by autoradiography, cut out with scissors, and counted in a liquid scintillation counter.

CYTOTOXICITY.—Flavonoids and related compounds used in this experiment did not cause platelet suspensions to release more than 7% of their lactate dehydrogenase at concentrations of 10^{-3}-10^{-6}M and, therefore, were not toxic to the cells (data not shown).

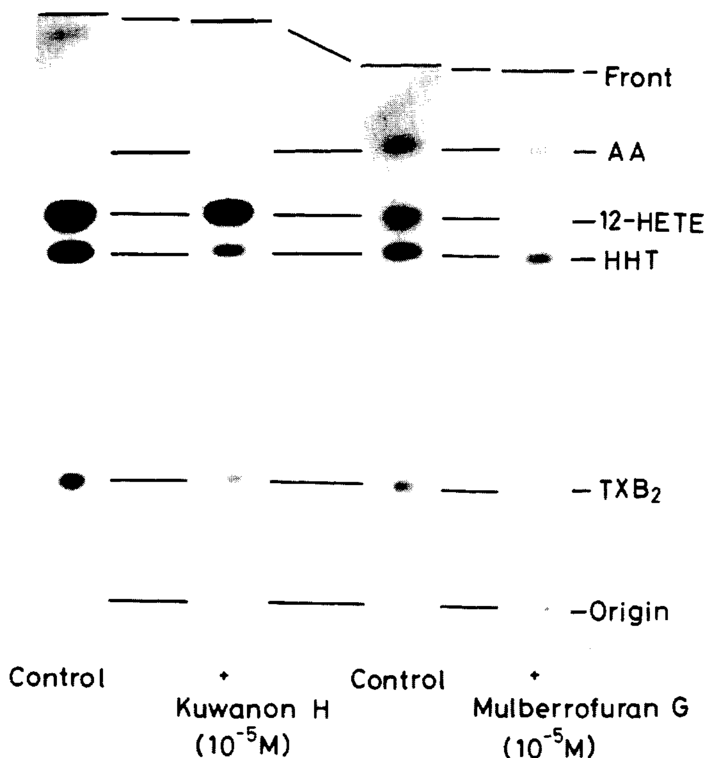

Figure 2. Autoradiograph of profiles on tlc of (1-^{14}C)arachidonic acid metabolism in rat platelets. Abbreviations: AA, arachidonic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; TXB_2, thromboxane B_2.

RESULTS

When arachidonic acid was incubated with rat platelets, it was converted to three main products: 12-HETE, HHT, and thromboxane B_2. The radioactivities of 12-HETE (12-lipoxygenase product), HHT, and thromboxane B_2 formed by the cyclooxygenase route in the control were 20.0±0.99, 17.4±0.43, and 18.0±0.44 ($\times 10^3$ cpm) (means ± standard errors for 27 experiments), respectively. The amounts of 12-HETE, HHT, and thromboxane B_2 formed after incubation for 5 min were found to be proportional to the amount of platelets present (data not shown).

Figure 2 shows the effects of kuwanon H and mulberrofuran G on the formation of 12-HETE, HHT, and thromboxane B_2 from (1-^{14}C)arachidonic acid in rat platelets. As seen from Figure 2, kuwanon H inhibited the formation of HHT and thromboxane B_2 without affecting the formation of 12-HETE. In contrast, mulberrofuran G inhibited the formation of 12-HETE without affecting the formation of HHT and thromboxane B_2. Kuwanons G and H, sanggenon C, and mulberrofuran Q caused dose-dependent inhibition of the formation of HHT and thromboxane B_2 and an increase in the formation of 12-HETE at concentrations of 10^{-5}-10^{-3}M. Sanggenon D and mulberrofuran J also inhibited the formation of HHT and thromboxane B_2, and at 10^{-4}M stimulated the formation of 12-HETE, but at 10^{-3}M they inhibited it. In contrast, mulberrofuran G caused a dose-dependent inhibition of the formation of 12-HETE; however, mulberrofuran G at concentrations of 10^{-4}-10^{-3}M inhibited the formation of HHT, thromboxane B_2, and 12-HETE. Kuwanon M and mulberroside A did not affect arachidonate metabolism in rat platelets. The results are summarized in Table 1.

DISCUSSION

The present investigation showed that various phenolic compounds isolated from

TABLE 1. Effects of Various Phenolic Compounds on Arachidonate Metabolism in Rat Platelets[a]

Compound	$IC_{50}(\times 10^{-5}M)$		
	12-HETE[b]	HHT	Thromboxane B_2
Kuwanon G(2,3)	E.E.	8.93	13.2
Kuwanon H(4)	E.E.	2.40	7.57
Kuwanon M(5)	*[c]	*[c]	*[c]
Sanggenon C(6)	E.E.	11.2	18.0
Sanggenon D(7)	*[d]	4.33	4.83
Mulberrofuran J (10)	82.3[e]	3.63	5.07
Mulberrofuran Q(12)	E.E.	22.7	35.5
Mulberrofuran G(8,9)	1.07	3.47	8.90
Mulberroside A (13)	N.E.	N.E.	N.E.

[a]Values are means for three experiments.

[b]Abbreviations: 12-HETE=12-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT=12-hydroxy-5,8,10-heptadecatrienoic acid; E.E.=enhancing effect; N.E.=no effect; * = >100.

[c]The percent activities of kuwanon M on the formation of HHT, thromboxane B_2, and 12-HETE are 68.0, 81.5, and 87.3%, respectively, at concentration of 10^{-3}M as compared to control values.

[d]The percent activities of sanggenon D on the formation of 12-HETE are 51.0% and 255.5% at concentrations of 10^{-3}M and 10^{-4}M, respectively, as compared to control values.

[e]The percent activities of mulberrofuran J on the formation of 12-HETE are 35.5% and 193.8% at concentrations of 10^{-3}M and 10^{-4}M, respectively, as compared to control values; not tested at concentrations higher than 10^{-2}M.

the rootbark of the cultivated mulberry tree significantly affected arachidonate metabolism in rat platelets. A number of nonsteroidal, anti-inflammatory drugs such as aspirin and indomethacin have been shown to inhibit cyclooxygenase enzymes but not to affect lipoxygenase enzymes (18-19). The thromboxane synthetase inhibitor OKY-1581 selectively inhibits the formation of thromboxane B_2 without inhibiting the formation of 12-HETE, and causes formation of PGD_2, PGE_2, and $PGF_{2\alpha}$ instead of thromboxane B_2 from arachidonic acid in platelets (25).

The phenolic compounds isolated from the rootbark of the mulberry tree may be grouped into four categories: (a)Diels-Alder type adducts of chalcones and dehydroprenyl-flavonoids, (b) Diels-Alder type adducts of chalcones and dehydroprenyl-2-arylbenzofurans, (c) Diels-Alder type adducts of prenylphenols and dehydroprenylphenols, and (d) stilbenes. Among the compounds used in this study, kuwanons G and H, sanggenon C, and mulberrofuran Q caused dose-dependent inhibition of the formation of HHT and thromboxane B_2 by the cyclooxygenase route, but dose-dependent stimulation of the formation of 12-HETE by the 12-lipoxygenase route. Sanggenon D and mulberrofuran J also produced a dose-dependent inhibition of the formation of HHT and thromboxane B_2, and at 10^{-4}M they enhanced the formation of 12-HETE, but at 10^{-3}M they inhibited its formation. These findings suggested that the 2,4-dihydroxyphenyl and 2,4-dihydroxybenzoyl moieties on the cyclohexene ring of group-A compounds such as kuwanons G and H, and sanggenons C and D, and group-B compounds such as mulberrofuran J, and a number of free phenolic hydroxy groups in the flavonoid and benzofuran skeletons may be essential for inhibiting of the formation of HHT and thromboxane B_2. The 2-arylbenzofuran derivative mulberrofuran Q also inhibited the formation of HHT and thromboxane B_2 without inhibiting the formation of 12-HETE. Mulberrofuran Q contains an epoxy ring. This epoxy ring may contribute to inhibition of the formation of HHT and thromboxane B_2. In contrast, the 2-arylbenzofuran, mulberrofuran G, produced a dose-dependent inhibition of the formation of 12-HETE. We previously reported that 2-arylbenzofurans such as mulberrofuran F with a 2,4-dihydroxy-3-γ,γ-dimethylallylphenyl moiety and a cyclohexane ring and albanol B with a 2,4-dihydroxyphenyl moiety had no effect (14). Furthermore, the 2-arylbenzofuran, mulberrofuran J with 2,4-dihydroxyphenyl and 2,4-dihydroxybenzoyl moieties on the cyclohexene ring at a concentration of 10^{-3}M inhibited the formation of 12-HETE. From these results, both the cyclohexane ring and the 2,4-dihydroxyphenyl moiety in mulberrofuran G may be required for its inhibitory effect on 12-lipoxygenase activity.

Nomura *et al.* reported that kuwanons G (2,3) and H (4) and sanggenons C (6) and D (7) of group-A, and mulberrofuran G (8,9) of group-B showed clear hypotensive effects. Thromboxane A_2 produced by platelets has strong effects in causing contraction of blood vessels (26) and inducing platelet aggregation (17). Moreover, at very low concentration, 12-HETE stimulates aortic smooth muscle cell migration (21). On the other hand, 12-HPETE produced by platelets inhibits collagen-induced platelet aggregation and inhibits the formation of thromboxane A_2 from arachidonic acid via the cyclooxygenase route in platelets (22). From the above results, it seems likely that phenolic compounds of mulberry tree could be developed in drugs to treat inflammatory diseases.

Experiments are now in progress to clarify the mechanisms and clinical significance of these effects.

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Received 24 January 1986