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Anti-atherosclerotic and anti-inflammatory activities of catecholic xanthones and flavonoids isolated from *Cudrania tricuspidata*

Ki Hun Park,[c] Yong-Dae Park,[a,b] Jong-Min Han,[a] Kyung-Ran Im,[a] Byong Won Lee,[c] Il Yun Jeong,[d] Tae-Sook Jeong[a,*] and Woo Song Lee[a,b,*]

[a]*National Research Laboratory of Lipid Metabolism and Atherosclerosis, Insect Resources Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 3806, Republic of Korea*
[b]*University of Science and Technology, Daejeon 305-333, Republic of Korea*
[c]*Division of Applied Life Science (BK21 Program), Department of Agricultural Chemistry, Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea*
[d]*Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Republic of Korea*

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Abstract—The catecholic xanthones and flavonoids **1**–**13** were isolated from the root bark of *Cudrania tricuspidata*. Compounds **1** and **3**–**8** exhibited significant antioxidant activity against low-density lipoprotein (LDL) oxidation in the thiobarbituric acid-reactive substance (TBARS) assay. Among them, prenylated flavonoids **10**–**12** showed an inhibitory effect on the NO production and iNOS expression in RAW264.7 cells. Also, compounds **1**, **2**, **5**, **7**, **9**, and **11** preferentially inhibited hACAT-2 than hACAT-1, whereas compounds **3**, **4**, **6**, and **8** showed a similar specificity against hACAT-1 and -2. However, flavonoids **10**, **12**, and **13** dominantly inhibited hACAT-2, not hACAT-1.

Low-density lipoprotein (LDL) and acyl CoA: cholesterol acyltransferase (ACAT) have been known to play a crucial role in the development of atherosclerosis and hypercholesterolemia.[1] Therefore, antioxidant against LDL oxidation and ACAT inhibitor are useful strategies for treating and/or preventing atherosclerosis and hypercholesterolemia. Antioxidants have exhibited antiatherogenic activities by inhibiting foam cell formation in animal models.[2] Among them, probucol and vitamin E are well-known as lipid-lowering agents and can lower coronary heart disease (CHD) risk.[3] However, both antioxidants of probucol and vitamin E showed the lowering effect of serum high-density lipoprotein (HDL) levels. On the other hand, many studies have been devoted to search rat liver microsomal ACAT inhibitor,[4] whereas the specific inhibitor against hACAT-1 or -2 has been studied rarely.[5] Recently, we reported that antioxidant, lignan or neolignan, against LDL oxidation and the specific inhibitor, saucerneol B, manassantin A and B, against hACAT-1 and -2 which were produced by Hi5 cells,[6] were isolated from the methanol extracts of *S. chinensis* root.[7] However, most studies have focused on developing antioxidant or ACAT inhibitor independently. Thus, we are interested in developing dual inhibitor against LDL oxidation and hACAT-1 or -2, from various plants.

Cudrania tricuspidata (Carr.) Bureau is one of the important traditional herbal remedies for anti-tumor, anti-inflammation, gastritis, and live damage in East Asian Countries such as Korea, China, and Japan.[8] Previous workers reported that root bark of this species contains biologically active isoprenylated xanthones[9] and flavonoids,[10] and also reported that extracts of root bark have been to be shown effective on high blood pressure,[11] inflammation, and cytotoxic to human tumor cell lines.[9g] Isoprenylated xanthones are known to have a variety of biological activities, such as hypertensive effect, anti-rhinoviral activity, inhibition of the formation of some prostanoids, and anti-tumor promoting activity.[9d] Recently, we reported that new or known catecholic xanthones **1**–**8** were isolated from root bark of

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* Corresponding authors. Tel.: +82 42 860 4278; fax: +82 42 861 2675 (W.S. Lee); tel.: +82 42 860 4588; fax: +82 42 861 2675 (T.S. Jeong); e-mail addresses: tsjeong@kribb.re.kr; wslee@kribb.re.kr

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C. tricuspidata, as shown in Figure 1. Some of them exhibited radical scavenging activities (e.g., DPPH, superoxide, and hydroxyl) and cytotoxic activities against human cancer cell lines such as HT-29, HL-60, SK-OV3, AGS, and A549.[12] Also, prenylated flavonoids were isolated from *C. tricuspidata* and exhibited antibacterial activity.[13] However, these catecholic xanthones **1**–**8** and prenylated falvonoids **9**–**13** were not evaluated for the antioxidant activity against LDL oxidation, NO production which was induced by an iNOS expression in RAW264.7 cells, and hACAT-1 or -2 inhibitory activities. In this study, we describe in vitro antioxidant activity against LDL oxidation, inhibition of NO production and iNOS expression in RAW264.7 cells, and human microsomal ACAT-1 or -2 inhibitory activities for catecholic xanthones **1**–**8** and prenylated flavonoids **9**–**13**.

Compounds **1**–**13** were isolated and evaluated in vitro for their potential to protect human LDL against Cu^{2+}-induced peroxidation.[14] The ability of compounds **1**–**13** to attenuate LDL oxidation was measured by measuring the amount of TBARS.[15] Effects of compounds **1**–**13** on production of TBARS were examined by incubating human LDL (120 μg/mL) in the presence of 10 μM $CuSO_4$ as an oxidation initiator. Compounds **1** and **3**–**8** exhibited potent antioxidant activities against LDL oxidation, as shown in Table 1. Then probucol was employed as a positive control. Among them, compound **4** bearing 5,6-dihydroxy group has shown the highest antioxidant activity against LDL oxidation, whereas compound **2** substituted with methoxy group at position 7 is not active. Compound **1** bearing 2,2-dimethylpyran ring was less effective than other compounds **3**–**8** which show relatively similar efficacy with probucol to be a positive control. Antioxidant activity for each compound is different more or less and it may be explained as due to their lipophilicity. Goto and his coworkers[16] have reported that α-tocopherol reduces the LDL oxidation 10- to 100-fold more potently than BHT or probucol in organic solution, but the difference in antioxidant activities against lipid peroxidation is smaller in the membranes due to their different lipophilicity. Also, compound **2** was not active as an antioxidant due to protection of C-7 hydroxyl group which acts as a reducing agent. In addition, prenylated flavonoids **9**–**13** were less effective than catecholic xanthones **1** and **3**–**8**, as shown in Table 1. Therefore, in order to act as an antioxidant, it can be rationalized that vicinal dihydroxy group could be converted to quinine easily by releasing two electrons as described in previous results.[12]

Also, the large amount of NO production produced by iNOS has been closely correlated with pathophysiology in atherosclerosis.[17] So, to determine the effects of compounds **9**–**13** on NO production in RAW 264.7 cells, the cells were treated with LPS (10 μg/mL) in the presence or absence of compounds **9**–**13** for 18 h. Then, the production of NO was measured by employing the method of Griess. LPS (10 μg/mL) increased the level of NO_2^- in culture medium by 10-fold as compared to control (LPS absence), whereas, among them, treatment of compounds **10** and **11** reduced NO production in 50 μM, whereas **12** inhibited NO production in a dose-dependent manner (Fig. 2A). Treatment of **10** and **12** at 80 μM and **11** at 50 μM did not affect cell viability as measured by the MTT method (data not shown). In order to measure the levels of iNOS protein which relates to produce inflammatory mediator NO, we examined iNOS protein expression levels by Western blot analysis. In contrast to control, treatment of compounds **10**–**12** at

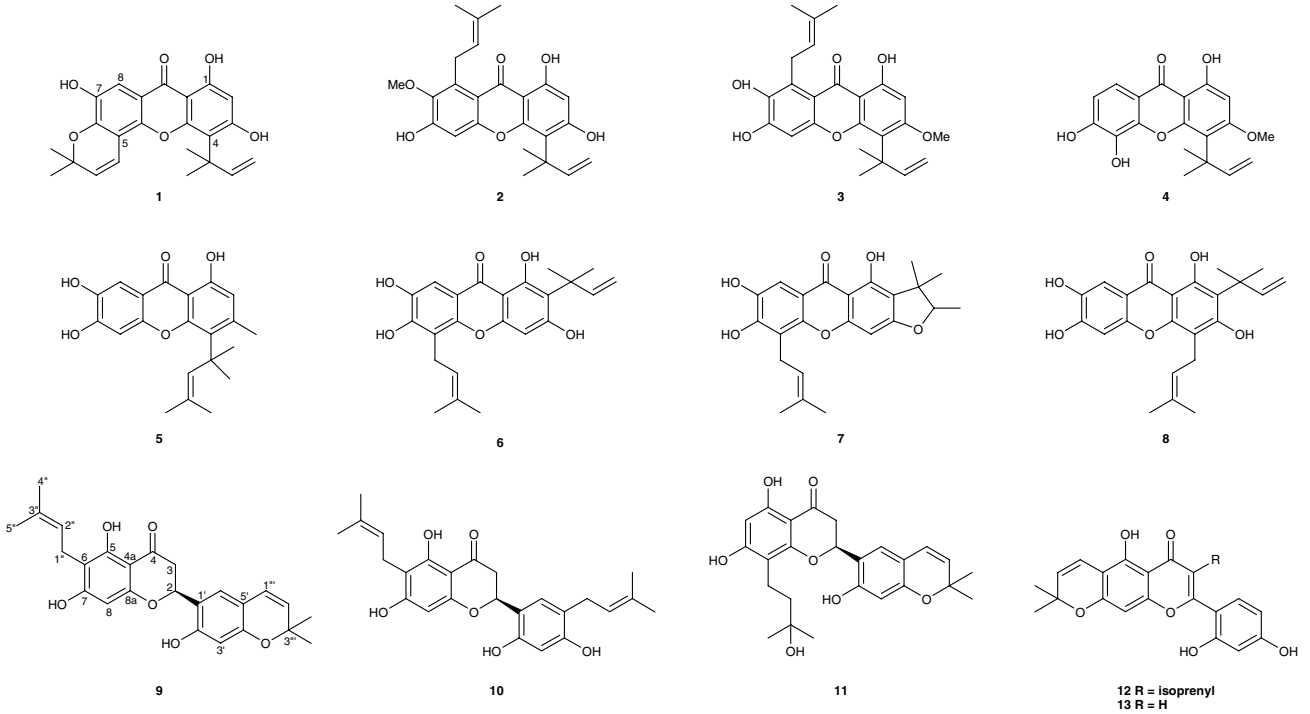

Figure 1. Chemical structures of **1**–**13** isolated from *C. tricuspidata*.

Table 1. Antioxidant activity against LDL oxidation and ACAT inhibitory activities of compounds **1**–**13**

Compound	LDL (IC_{50}, μM)[a]	hACAT-1 (IC_{50}, μM)[b]	hACAT-2 (IC_{50}, μM)[b]
1	12.6	40	28.8
2	NA	20	19.2
3	6.2	57.6	75.2
4	0.8	148	132.8
5	2.6	89.6	41.6
6	3.8	68.0	74.4
7	2.2	56.8	23.2
8	4.5	96.0	112.0
9	43.2	82.4	24.8
10	65.6	NA	89.6
11	27.2	59.2	44.8
12	46.4	NA	41.6
13	62.4	NA	77.6
Probucol[c]	3.6		
Oleic acid anilide[d]		0.14	0.17

[a] In vitro antioxidant activity was measured using human plasma LDL (120 μg/mL). Data are shown as mean values of two independent experiments performed in duplicate.
[b] In vitro ACAT inhibitory activity was measured using the expressed hACAT-1 or hACAT-2. Data are shown as mean values of two independent experiments performed in duplicate.
[c] Probucol was used as a positive control.
[d] Oleic acid anilide was used as a positive control.

5, 10, 20, and 50 μM, respectively, suppressed markedly the induction levels of iNOS protein (Fig. 2B).[18] As a result, NO production in RAW 264.7 cells was also inhibited by **10**–**12** at a similar order of activity to that obtained in copper-induced LDL-oxidation.

The potential of compounds **1**–**13** was first evaluated as inhibitor of hACAT-1 or -2 that was expressed and characterized from infected Hi5 cells by recombinant baculoviruses.[6] Then, the rate of incorporation of [1-^{14}C]oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT-1 or -2. With expecting to show different inhibitor specificity against hACAT-1 and -2, compounds **1**–**13** were applied to the enzyme assay employing each human ACAT isoform. Compounds **1**, **2**, **5**, **7**, **9**, and **11** preferentially inhibited hACAT-2 than hACAT-1, whereas compounds **3**, **4**, **6**, and **8** showed a similar specificity against hACAT-1 and -2. However, flavonoids **10**, **12**, and **13** dominantly inhibited hACAT-2, not hACAT-1. Then, the ACAT inhibitory activities of the compounds **1**–**13** were confirmed by the positive control with oleic acid anilide, which inhibited hACAT-1 and hACAT-2 with IC_{50} values of 0.14 μM and 0.17 μM, respectively (Table 1).[6] According to recent results, manassantin A showed more specific inhibitory activity against hACAT-2 compared to hACAT-1, whereas manassantin B dominantly inhibited hACAT-1. On the other hand, pyripyropene A inhibited only hACAT-2.[7b] Previously, Lada et al. described that some regions of either enzyme were on opposite sides of the membrane, suggesting uniqueness of function for ACAT-1 and ACAT-2.[5] The cellular localization of ACAT-1 and ACAT-2 also was unique. The results in the present study again suggest uniqueness of function for ACAT-1 and ACAT-2.

In conclusion, the catecholic xanthones **1**–**8** and flavonoids **9**–**13** were isolated from the chloroform extracts of the root bark of *C. tricuspidata*. Compounds **1** and **3**–**8** exhibited significant antioxidant activity against LDL oxidation in TBARS assay. Among them, **10**–**12** showed an inhibitory effect on the NO production and iNOS expression in RAW264.7 cells. Also, compounds **1**, **2**, **5**, **7**, **9**, and **11** preferentially inhibited hACAT-2 than hACAT-1, whereas compounds **3**, **4**, **6**, and **8**

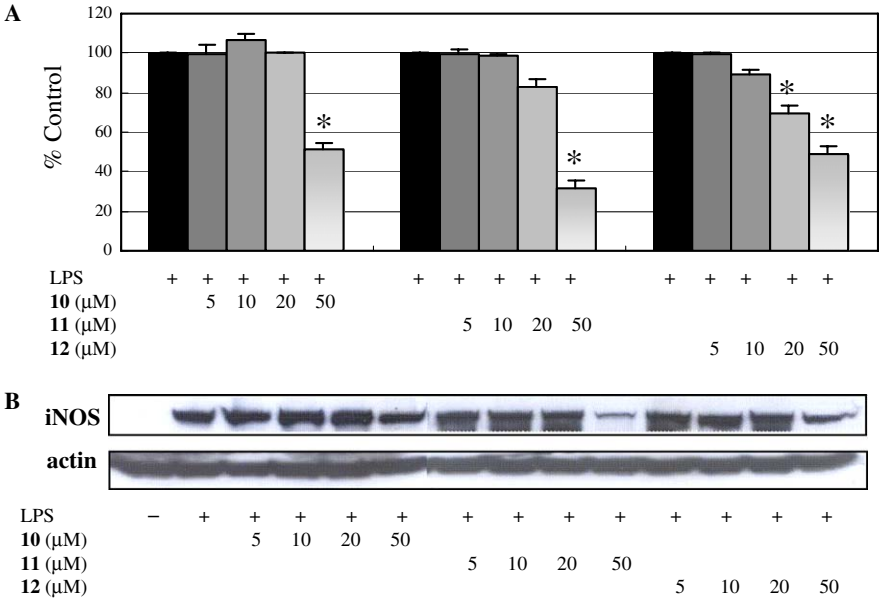

Figure 2. Effects of **10**–**12** on the induction of iNOS by LPS. (A) Inhibition of NO production by **10**–**12** in RAW264.7 cells. RAW264.7 cells were treated with various concentrations of **10**–**12** dissolved in dimethylsulfoxide for 2 h prior to the addition of LPS (10 μg/mL), and the cells were further incubated for 18 h. (B) Inhibition of LPS-inducible iNOS protein expression by **10**–**12**. The level of iNOS protein was monitored 18 h after treatment of cells with LPS (10 μg/mL). Values are means ± SEM ($n = 2$). $^{*}P < 0.01$ versus LPS alone.

showed a similar specificity against hACAT-1 and -2. However, flavonoids **10**, **12**, and **13** dominantly inhibited hACAT-2, not hACAT-1.

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18. *Cell culture.* RAW264.7 cells (murine macrophage cell line) obtained from American Type Culture Collection (Manassas, VA) were cultured in DMEM (Gibco-BRL, Grand Island, NY, USA) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS in a humidified incubator with 5% CO_2/95% air at 37 °C. After incubation for 18 h, cells were pretreated with compounds **10**–**12** for 2 h, followed by incubation with LPS (10 μg/mL). *Nitric oxide (NO) determination.* NO_2^- accumulation was monitored by measuring the nitrite content in culture medium. RAW264.7 cells were plated at 1×10^6 cells/mL and stimulated with LPS in the presence or absence of **10**–**12** for 18 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring the optical density at 540 nm with ELISA reader (Bio-RAD, Hercules, CA, USA). *Western Blot.* Cell lysates were prepared by suspending 1×10^6 cells in 100 μL lysis buffer (140 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.5), disrupted by sonication, and extracted at 4 °C for 30 min. The extracts were centrifuged at 15,000*g* for 10 min. Equal amounts of proteins were separated on a 10% SDS–PAGE and then transferred to Immobilon-P membrane (Millipore co., Bedford, MA, USA). The membrane was incubated for 2 h at room temperature with iNOS antibody (BD Transduction Laboratories, Lexington, KY, USA). Immunoreactive proteins were detected with the ECL Western blotting kit.