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Kinetics of Radical-Scavenging Activity of Hesperetin and Hesperidin and their Inhibitory Activity on COX-2 Expression

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Abstract. *The radical-scavenging activities of the flavanones hesperetin and hesperidin were investigated by differential scanning calorimetry (DSC) monitoring of the polymerization of methyl methacrylate initiated by 2,2'-azobisisobutyronitrile (AIBN, an R• radical) or benzoyl peroxide (BPO, a PhCOO• radical) at 70˚C under nearly anaerobic conditions. Their stoichiometric factor (number of free radicals trapped by one mole of antioxidant moiety (n)) and the ratio of the rate constant of inhibition to that of propagation (k_{inh}/k_p) were determined and compared with that for trolox. The n value declined in the order trolox (2.0) > hesperetin (0.8) > hesperidin (0.2) in the AIBN system, whereas it declined in the order hesperetin (0.9) > trolox (0.1) > hesperidin (0.0) in the BPO system. The k_{inh}/k_p value declined in the order hesperidin (195) > hesperetin (33) > trolox (12) in the AIBN system, whereas it declined in the order hesperidin (362) > trolox (127) > hesperetin (18) in the BPO system. The n value of about 1 for hesperetin with a relatively small k_{inh}/k_p value suggests the formation of dimers, as a result of the coupling reaction of phenolic monomers. In contrast, n values << 1 for hesperidin and trolox in the BPO system resulted in very high values for k_{inh}/k_p. Hesperidin was also much more able to suppress the growth of methyl methacrylate radicals, although its n value was small, suggesting that this compound may also suppress polyunsaturated fatty acid radicals. In the concentration range 250-500 μM, hesperetin and hesperidin showed potent inhibition of LPS-induced expression of the COX-2 gene in RAW 264.7 cells, suggesting the anti-inflammatory activity of these compounds. The ability of hesperetin and hesperidin to suppress COX-2 gene expression may be a consequence of their antioxidant activity.*

We have recently reported that dimerization of mono-methoxyphenols is capable of increasing their antioxidant and anti-inflammatory activities (1, 2). In RAW 264.7 cells, dehydrodiisoeugenol, a dimer of iso-eugenol and bis-eugenol, a dimer of eugenol, strongly inhibited LPS-stimulated nuclear-factor-kappa-B (NFÎ B) activation and cyclooxygenase *(COX)-2* gene expression, both of which are closely involved in inflammation and mutagenesis. Thus, it is probable that the flavanones hesperetin and hesperidin possess anti-inflammatory activity. Hesperidin was previously reported to inhibit tumor promotion in a two-stage skin tumorigenesis protocol in CD-1 mice and also to prevent 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced edema in CD-1 mouse ear (3). Methoxy derivatives such as curcumin show strong inhibition of TPA-induced ornithine decarboxylase activity in mouse skin (4). The potential to suppress COX-2 and iNOS gene expression has previously been reported to be correlated with the antioxidant activities of curcumin derivatives (5). Methoxyphenols, with strong free radical-scavenging activity, may suppress LPS-induced gene expression partly by reducing the oxidative stress caused by LPS treatment, and this mechanism may be responsible for the inhibition of AP-1 and NFÎ B DNA-binding activity (6). Thus, it is of interest to investigate a possible link between the anti-inflammatory and antioxidant activities of methoxyphenols.

Some studies of the antioxidant activity of flavanone glycosides (hesperidin) and their aglycones (hesperetin) have previously been reported (7-12), but the kinetics of the radical-scavenging activity of flavanones remain obscure. We have previously reported the use of differential scanning calorimetry (DSC) to evaluate the radical-scavenging activities of ferulic acid (13), curcumin (14) and various phenols (15) by the induction period method. The antioxidant activity of these compounds was predicted well by a model based on kinetic and thermodynamic data.

In the present study, the radical-scavenging activity of hesperetin and hesperidin was investigated by using the induction period method with DSC monitoring. The inhibitory effect of these flavanones on LPS-stimulated COX-2 expression in RAW 264.7 cells was also investigated.

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Key Words: Hesperetin, hesperidin, radical-scavenging activity, inhibition rate constants, COX-2.

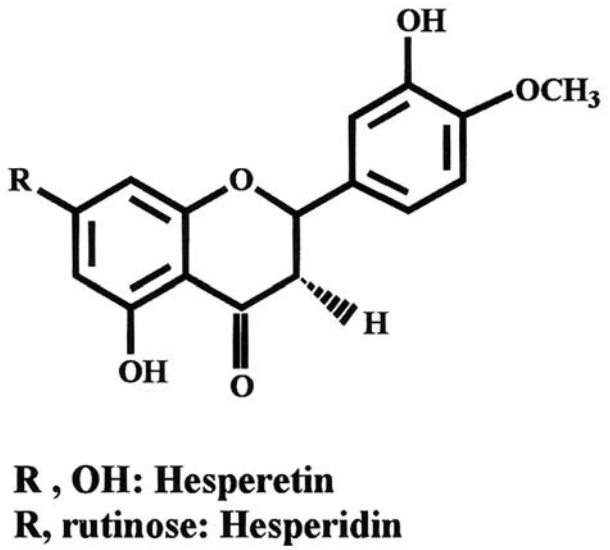

Figure 1. *Chemical structures of the flavanones hesperetin and hesperidin.*

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies. Hesperetin (3',5,7-trihydroxy -4-methoxyflavanone), hesperidin (hesperetin 7-rhamnoglucoside) (Sigma Chemical Co., St. Louis, MO, USA); methyl methacrylate (MMA) (Tokyo Kasei Kogyo Co., Tokyo, Japan.); 2,2'-azobisisobutyronitrile (AIBN), benzoyl peroxide (BPO), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). AIBN and BPO were recrystallized from methanol and chloroform/methanol (1:1 v/v), respectively. The chemical structures of hesperetin and hesperidin are shown in Figure 1.

Megaprime DNA labeling system, 5'-end labeling system and 5'-[α-^{32}P]dCTP (Amersham Biosciences Co., Piscataway, NJ, USA; RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA); FBS (HyClone, Logan, UT, USA); *Escherichia coli* O111 B4-derived LPS (List Biological Laboratories, Inc., Campbell, CA, USA); [4,5-dimethylthiazo-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co.); RAW 264.7 cells (Riken Cell Bank, Saitama, Japan); COX-2 cDNA probes (Cayman Chemical Co., Ann Arbor, MI, USA); plasmid containing β-actin cDNA (Japanese Cancer Research Bank, Tokyo, Japan).

Induction period method. The induction period method was used to determine the inhibition rate (R_{inh}) due to the thermal decomposition of AIBN or BPO (12-15), using Equation **(1)**,

$$R_i = n\,[IH]_0/[IP] \qquad \textbf{(1)}$$

where $[IH]_0$ is the concentration of the inhibitor at time zero, and [IP] is the induction period. The R_i values of AIBN and BPO were 5.66×10^{-6} and 2.28×10^{-6} M s^{-1}, respectively, with 2, 6-di-*t*-butyl-4-methoxyphenol as an inhibitor (12).

Induction period and inhibition rate of polymerization. The induction period and initial rate of polymerization were determined by the method previously reported (12-15). In brief, the resin consisted of MMA and AIBN or BPO, with or without additives. AIBN or BPO was added at 1.0 mol%, and the additives were used at 0.001, 0.01, 0.02, 0.04 and 0.05 mol%. Approximately 10 μl of the experimental resin (MMA: 9.12-9.96 mg) was loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) kept at 70˚C, and the thermal changes induced by polymerization were recorded for the appropriate periods. The conversion (polymer yield) of all samples, as calculated from DSC thermograms, was 92-96%. Polymerization curves were derived from DSC thermograms using the integrated heat evoked by the polymerization of MMA. Time-exotherm and time-conversion curves of hesperetin (A) and hesperidin (B) and of trolox for the AIBN system and for the BPO system are shown in Figures 2 and 3, respectively. Polymerization curves break when an inhibitor is consumed. These breaks are sharp and provide a reliable measure of the induction period of the inhibitor. The presence of oxygen retards polymerization, because oxygen reacts with MMA radicals activated by the initiator and then subsequently produces a non-radical product. Thus, polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen since it had been sealed in air. Tangents were drawn to polymerization curves at an early stage in the run. The induction period of the test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of tangents drawn to the early stage of polymerization. The induction period (IP) was calculated from the difference between the induction period of specimens and that of controls. The initial rates of polymerization in the absence (R_p) and presence (R_{inh}) of the indicated compounds were calculated from the slopes of the first linear portions of plots of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage).

The initial rate of polymerization in the presence of an inhibitor [IH] is given by Equation **(2)** (16, 17),

$$R_{inh} = k_p[MMA]R_i/nk_{inh}[IH] \qquad \textbf{(2)}$$

where k_{inh} is the inhibition rate constant.

Equations **1** and **2** give Equation **(3)**,

$$k_{inh}/k_p = [MMA]/(R_{inh}\,[IP]) \qquad \textbf{(3)}$$

Cell culture. Cells of the murine macrophage cell line RAW 264.7 were cultured to the subconfluent state in RPMI 1640 medium supplemented with 10% FBS at 37˚C under 5% CO_2 in air, washed, and then incubated overnight in serum-free RPMI 1640. They were then washed further and treated with the test samples.

Plasmid preparation. The methods used for plasmid preparation have been previously described (18).

Northern blot analysis. RAW 264.7 cells (10^6 cells/dish) in Falcon 5-cm-diameter dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were treated with the test samples and their total cellular RNA was then extracted by the AGPC procedure (19). As described earlier (1), the RNA was subjected to 1% agarose electrophoresis and blotted onto nylon membranes (Micron Separations, Inc., Westboro, MA, USA). The membranes were then hybridized with cDNA probes that had been labeled with 5'-[α-^{32}P]dCTP by use of a Megaprime DNA labeling system. After hybridization, the membranes were washed, dried and exposed overnight to Kodak X-ray film (Eastman Kodak Co., Rochester,

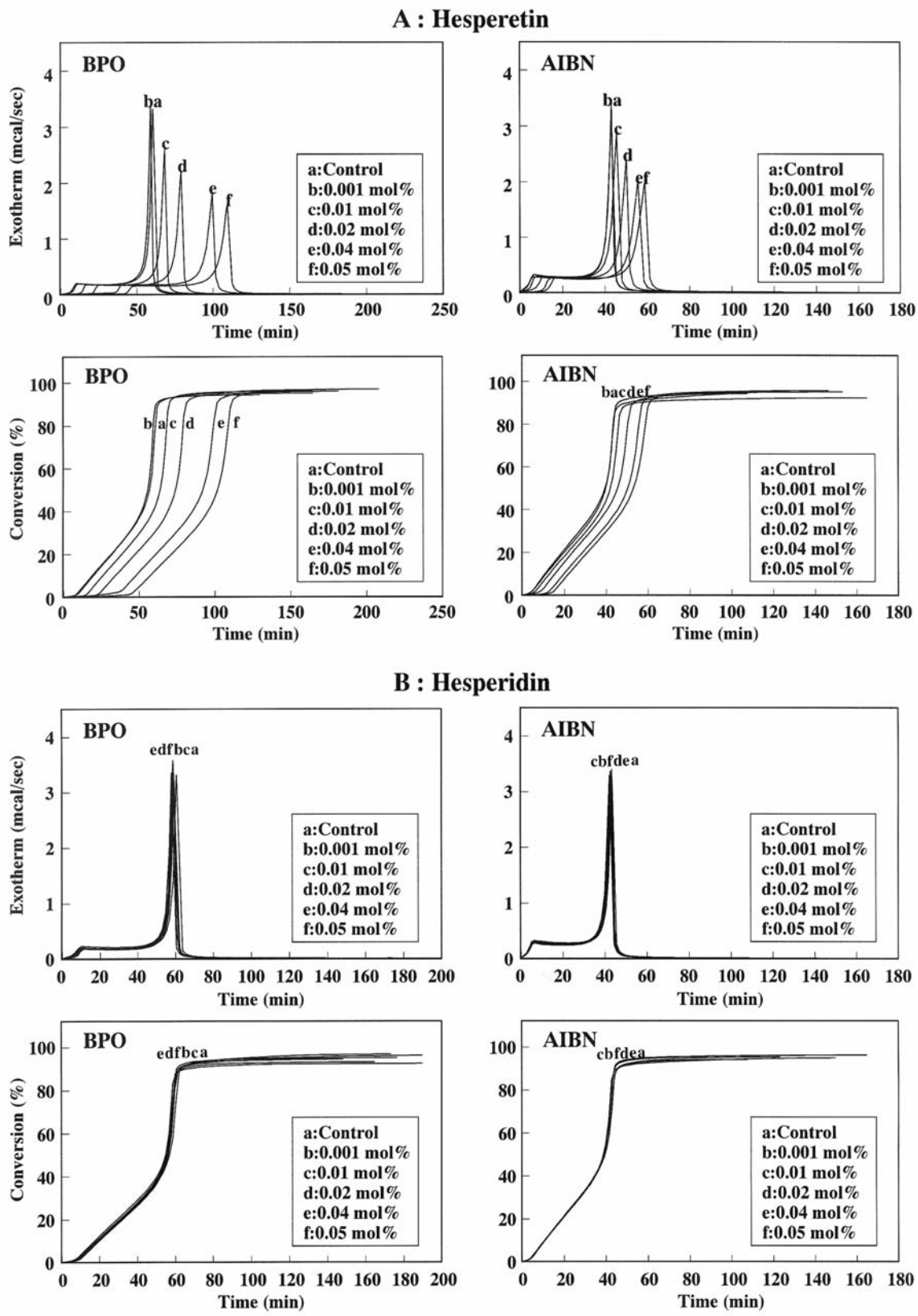

Figure 2. *Exothermic (top) and time-conversion (bottom) curves for the polymerization of methyl methacrylate initiated by the thermal decomposition of AIBN and BPO in the presence of hesperetin (A) and hesperidin (B).*

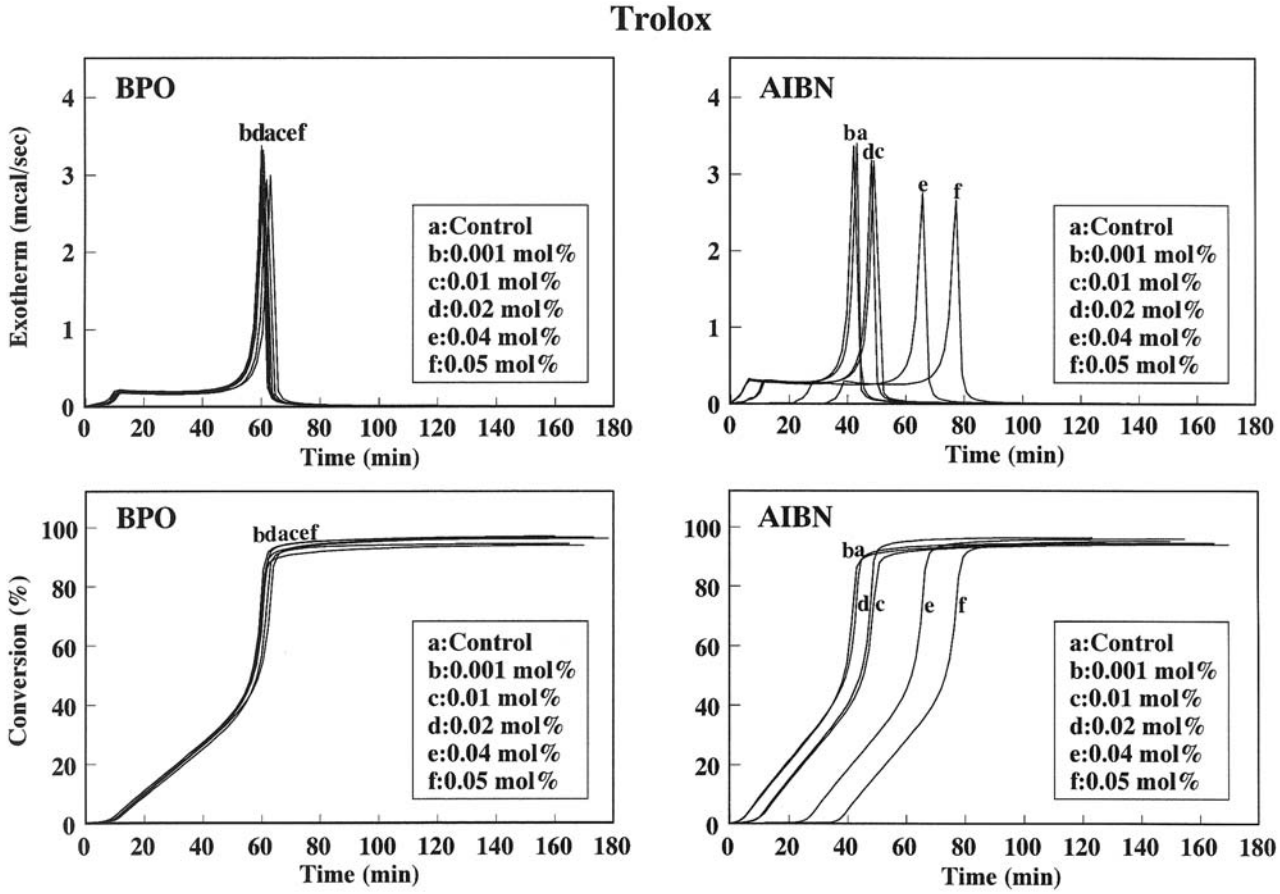

Figure 3. *Exothermic (top) and time-conversion (bottom) curves for the polymerization of methyl methacrylate initiated by the thermal decomposition of AIBN and BPO in the presence of trolox.*

NY, USA) at –70˚C. β-Actin was used as internal standard for quantification of total RNA in each lane of the gel.

Determination of cell viability. RAW 264.7 cells ($2.5x10^4$ cells/well) were plated in 96-well plates. After treatment with the indicated compounds for 2 h, 20 μl of MTT (5 μg/ml) was added to each well, and incubation was continued for an additional 4 h. The relative cell number was determined by measuring the absorbance at 540 nm of the cell lysate with a Labsystem Multiskan (Bichromatic Labsystem, Helsinki, Finland) with a Star/DOT Matrixprinter JL-10. The 50% toxic concentration was determined from the dose-response curve.

Results and Discussion

Radical-scavenging activity. Time-exotherm (top) and time-conversion (bottom) curves for hesperetin (A) and hesperidin (B) (Figure 2) show that the radical-scavenging activity of hesperidin was markedly different from that of hesperetin. The activity of hesperidin in both the AIBN and BPO systems was not enhanced at concentrations above 0.01 mol%. Similarly, the radical-scavenging activity of trolox in the BPO system was not greatly enhanced by increasing its concentration, whereas a marked increase in activity with increasing concentration was apparent in the AIBN system (Figure 3). Plots of IP *vs.* concentrations in the AIBN (a) and BPO system (b) are shown in Figure 4. The linear relationships for each compound was found. IP, n, R_{inh}/R_p and k_{inh}/k_p values for each antioxidant are summarized in Table I. The IP in the AIBN system declined in the order trolox > hesperetin > hesperidin, whereas that in the BPO system declined in the order hesperetin > trolox > hesperidin. In both systems hesperetin showed an n value of about 1, suggesting that this compound may undergo dimerization as a result of a radical coupling reaction. We previously used a semi-empirical PM 3 calculation to suggest the formation of dimers of hesperetin (12). The n for hesperidin in both systems was close to 0. Trolox (a water-soluble analog of vitamin E) is well known as an

antioxidant. Trolox inactivates alkylperoxy and superoxide radicals in natural media (20). In the present study, the *n* value for trolox in the AIBN system was 2, whereas that in the BPO system was 0.1. Cyanoisopropyl radicals ($R^{\bullet}$) derived from AIBN immediately react with molecular oxygen contained in the DSC pan and consequently are converted to $ROO^{\bullet}$ radicals. However, after molecular oxygen is completely consumed, radicals derived from AIBN are mainly alkyl radicals ($R^{\bullet}$). On the other hand, the radicals derived from BPO are $PhCOO^{\bullet}$, and the small quantity of molecular oxygen contained in the DSC pan is probably capable of reacting with substrates such as trolox during the induction period. Thus, the lower *n* value for trolox in the BPO system could be caused by an interaction between trolox and molecular oxygen, because trolox, like α-tocopherol, is sensitive to oxygen as a biradical.

The R_{inh}/R_p value for hesperidin was lower than that for the other compounds, suggesting potent suppression of growing MMA radicals. Furthermore, the k_{inh}/k_p value for hesperidin was the highest among the compounds tested. Although hesperidin had the lowest *n* value, its inhibition rate constant was very high. This suggests that, in biological systems, hesperidin may be a strong suppressor of polyunsaturated fatty acid radicals derived from reactive oxygen species.

The k_{inh} value for each compound was estimated from the value of the rate constant of propagation of MMA (k_p=405 M^{-1} s^{-1} at 70˚C, as extrapolated from values of 143 M^{-1} s^{-1} at 30˚C and 367 M^{-1} s^{-1} at 60˚C (21)). From the k_{inh}/k_p values for each compound shown in Table I, the k_{inh} values for hesperetin, hesperidin and trolox in the AIBN system were $1.34x10^4$ M^{-1} s^{-1}, $7.94x10^4$ M^{-1} s^{-1} and $0.48x10^4$ M^{-1} s^{-1}, respectively. In contrast, in the BPO system, the corresponding values were $0.73x10^4$ M^{-1} s^{-1}, $14.66x10^4$ M^{-1} s^{-1} and $5.14x10^4$ M^{-1} s^{-1}, respectively. Among the compounds tested, hesperidin showed the largest value of k_{inh} (about $10x10^4$ M^{-1} s^{-1}) in both systems. The k_{inh} for hesperetin was similar in both systems, in the range of $0.73x10^4$ M^{-1} s^{-1} to $1.34x10^4$ M^{-1} s^{-1}, whereas the k_{inh} for trolox in the BPO system was ten times greater than that in the AIBN system. The k_{inh} values of hesperetin, hesperidin and trolox with superoxide radicals have previously been reported to be $5.9x10^3$ M^{-1} s^{-1}, $2.8x10^4$ M^{-1} s^{-1} and $5.8x10^3$ M^{-1} s^{-1}, respectively (7). Again, hesperidin showed the largest k_{inh} value, followed by hesperetin and trolox. Other studies have provided a k_{inh} value for trolox reacting with superoxide of $4.9x10^3$ M^{-1} s^{-1} (20) and for trolox reacting with $ROO^{\bullet}$ of $11x10^4$ M^{-1} s^{-1} (17). The k_{inh} is, therefore, dependent on the radical species, since trolox shows a greater reactivity with $ROO^{\bullet}$ radicals than with superoxide radicals. In the present study, for each compound the k_{inh} for the reaction with poly-MMA radicals initiated by $PhCOO^{\bullet}$ radicals was greater than that for the reaction with poly-MMA radicals initiated by $R^{\bullet}$ radicals.

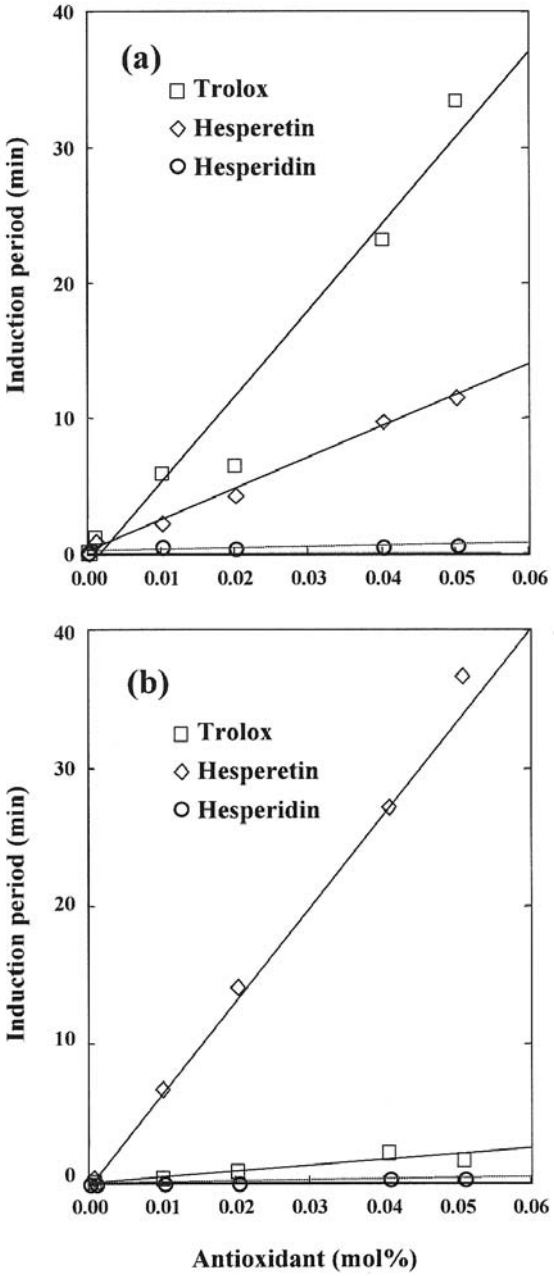

Figure 4. *Plots of induction period vs. concentrations of hesperetin, hesperidin and trolox in the polymerization of methyl methacrylate initiated by thermal decomposition of AIBN (a) and BPO (b).*

Hesperetin, derived from the hydrolysis of the aglycone, and hesperidin have previously been reported to be capable of scavenging peroxynitrite ($ONOO^-$) (8), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and reactive oxygen species (ROS) (10). Hesperetin has been recognized to have stronger activity than hesperidin (8, 10), which was consistent with results of *n* values in the

Table I. *Radical scavenging activities for hesperetin, hesperidin and trolox.*

System	Phenol	IP(s)	R_{inh} x 10^3 (M s^{-1})	R_p/R_{inh}	k_{inh}/k_p	*n*
AIBN-MMA	Hesperetin	137	2.1	1.00	33	0.8
AIBN-MMA	Hesperidin	37	1.3	0.96	195	0.2
AIBN-MMA	Trolox	351	2.2	1.00	12	2.0
BPO-MMA	Hesperetin	413	1.3	0.97	18	0.9
BPO-MMA	Hesperidin	20	1.3	0.96	362	0.04
BPO-MMA	Trolox	53	1.4	1.00	127	0.1

Methyl methacrylate (MMA), 9.4 M; AIBN (or BPO)1 mol%; phenol, 0.01 mol%; Induction period (IP)=(IP_{sample}–IP_{cont}); initial rates of polymerization in the absence and presence of a phenol are R_p and R_{inh}, respectively. The rate constants of inhibition and propagation are K_{inh} and K_p, respectively. Stoichiometric factor (*n*); at 70˚C. Values are means for three different experiments. Standard errors are <5%.

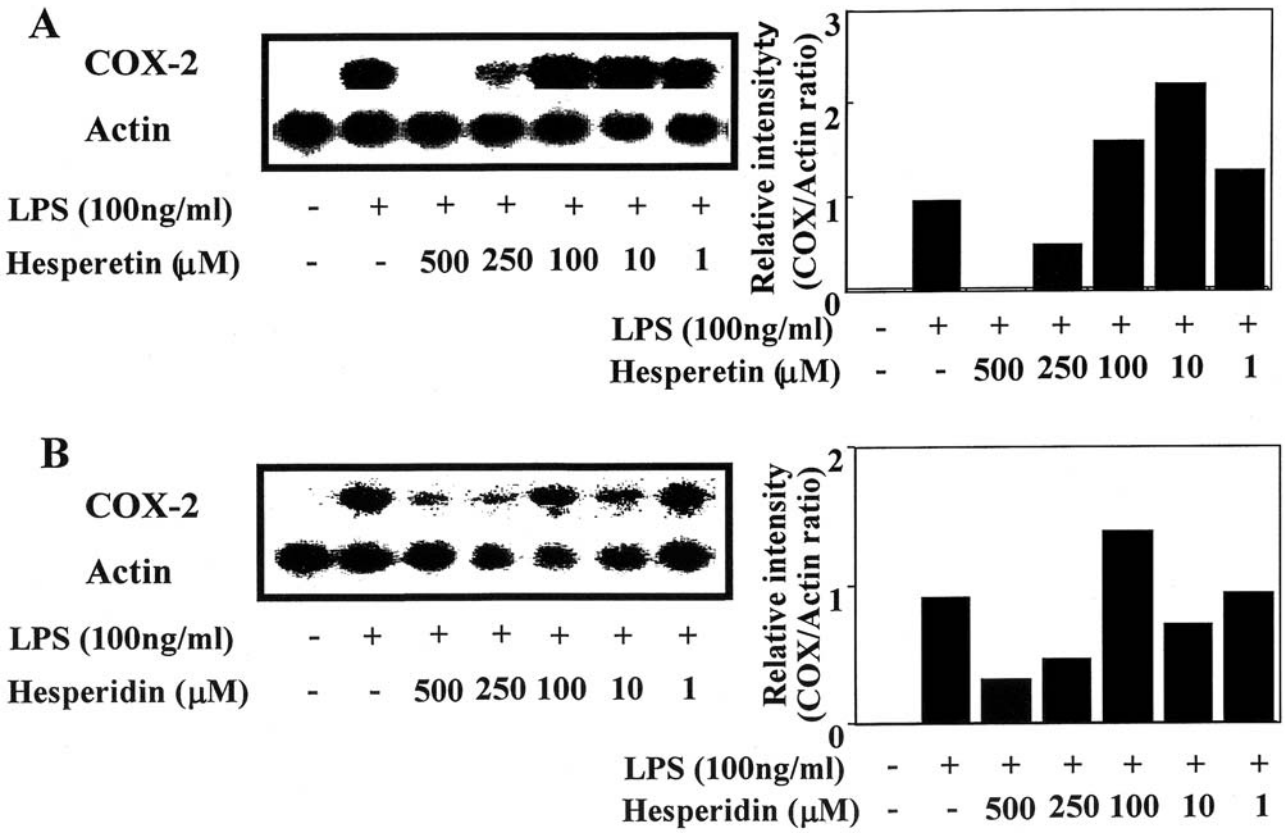

Figure 5. *Regulatory effect of hesperetin (A) and hesperidin (B) on LPS-induced expression of the COX-2 gene in RAW264.7 cells. The cells were pretreated or not for 30 min with the indicated dosages of hesperetin and hesperidin. They were then treated or not with LPS at 100 ng/ml. At 3 h after addition of LPS, total RNA was isolated and Northern blot analysis was performed with COX-2 and β-actin cDNAs as probes. Right panels: quantification of expression of COX-2 as shown in the left panels was performed by densitometry; and the data are expressed as relative signal intensity (COX-2/β-actin ratio). Bars represent the means for 3 independent experiments. Standard errors were <15%. There is a significant difference between controls and hesperetin at 250 μM ($p<0.01$) and 500 μM ($p<0.001$). There is also a significant difference between controls and hesperidin at 250 μM and 500 μM ($p<0.01$).*

present study. However, although the *n* value for hesperidin was small in the present study, its great k_{inh} value suggested that this compound is also a potent antioxidant in a propagation process. It has been suggested that hesperetin promotes cellular defence activation in the protection against $ONOO^-$-involved diseases, such as Alzheimer's disease, rheumatoid arthritis, cancer and other inflammatory conditions (8).

Inhibitory effect on COX-2 expression. The rate-limiting step in the conversion of arachidonic acid to prostaglandin H2, the precursor of biologically active PGs, is catalyzed by the enzyme COX. In a variety of cell types, COX-2 is induced by growth factors, cytokines and LPS *via* activation of transcription factors such as NFÎ B. COX-2 is closely involved in inflammation, arthritis, Alzheimer's disease, pain and cancer (22-26). The Northern blot assay (Figure 5) showed that the LPS-induced gene expression of COX-2 was clearly inhibited by hesperetin and hesperidin at a concentration of 250 μM, suggesting that these compounds possess an anti-inflammatory effect. The activity of hesperidin was similar to that of hesperetin, although the k_{inh} for the former was greater than that for the latter. The inhibitory effect of hesperidin supports previously published data showing the potent anti-inflammatory activity of this compound (3). Hesperetin and hesperidin do not cause cell death at concentrations up to 500 μM. The 50% lethal concentration for both compounds was about 500 μM.

Curcumin, a methoxyphenol, was previously reported to significantly inhibit LPS-induced COX-2 expression in RAW 264.7 cells at a concentration of 20 μM (27). The ability of curcumin to suppress cancer development and inflammatory activity is well established (28). Hesperetin and hesperidin may also possess anticancer activity.

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