Radical-scavenging and Anti-inflammatory Activity of Quercetin and Related Compounds and Their Combinations Against RAW264.7 Cells Stimulated with *Porphyromonas gingivalis* Fimbriae. Relationships between Anti-inflammatory Activity and Quantum Chemical Parameters

YUKIO MURAKAMI[1], AKIFUMI KAWATA[1], SHIGERU ITO[2], TADASHI KATAYAMA[1] and SEIICHIRO FUJISAWA[1]

[1]*Department of Diagnostic and Therapeutic Sciences, Division of Oral Diagnosis and General Dentistry, Meikai University School of Dentistry, Sakado City, Saitama, Japan;*
[2]*Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-Ku, Tokyo, Japan*

Abstract. *Background/Aim: The flavonoid quercetin exerts significant anti-inflammatory activity against chronic infections, including periodontal disease. However, it is unclear whether combination of quercetin with other flavonoids enhances antioxidant and anti-inflammatory activity. To clarify the molecular mechanism responsible for the anti-inflammatory activity of quercetin, we investigated the antioxidant, cytotoxicity and anti-inflammatory activity of quercetin and its related compounds, catechin and epicatechin, and their combinations. Materials and Methods: Radical-scavenging activities were determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and cytotoxicity against RAW264.7 cells was determined using a cell counting kit (CCK-8). The inhibitory effects of these compounds on the mRNA expression of cyclooxygenase-2 (Cox2), tumor necrosis factor-alpha (Tnfα) and nitric oxide synthase 2 (Nos2), in RAW264.7 cells stimulated with Porphyromonas gingivalis (Pg) fimbriae, was also determined using real-time polymerase chain reaction analysis. The phenolic O-H bond dissociation enthalpy (BDE) and quantum chemical parameters were calculated on the basis of density function theory (DFT) BLYP/6-31G*. Results: The DPPH• radical-scavenging activity (EC_{50}) of*

quercetin, catechin and epicatechin was 5.5, 7.7 and 6.2 μM, respectively, whereas the cytotoxicity (LC_{50}) was 4.45, 4.80 and 4.95 mM, respectively. Quercetin had slightly higher cytotoxicity and anti-DPPH• activity than catechin and epicatechin. The BDE for the three flavonoids at the 4'-OH in the B ring, which is the initial active site, was about 75 kcal/mol. Furthermore, various combinations of quercetin with catechin or epicatechin exerted an antagonistic effect on anti-DPPH• activity. Gene expression of Cox2, Tnfα and Nos2 stimulated by exposure to Pg-fimbriae was markedly suppressed by quercetin, but was not modulated by its combination with epicatechin. The 50% inhibitory concentration of quercetin for Cox2 expression was approximately 10 μM, while that of catechin and epicatechin was approximately 500 μM. Values of the quantum chemical parameters softness (σ) and electronegativity (χ) were highest for quercetin among the three flavonoids tested. Conclusion: The potent anti-inflammatory activity of quercetin appears to be attributable to its high σ and χ values. Quercetin may be applicable as a preventive agent against inflammatory periodontal disease as a manifestation of systemic disease.

Correspondence to: Dr. Yukio Murakami, Division of Oral Diagnosis and General Dentistry, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado City, Saitama 350-0283, Japan. Tel: +81 492855511, Fax: +81 492876657, e-mail: ymura@dent.meikai.ac.jp

Conditions such as diabetes mellitus (1), cardiovascular disease (2) and preterm low birth weight (3) are associated with an increased risk of chronic infections such as periodontitis. *Porphyromonas gingivalis* (Pg) is a key bacterium strongly associated with early-onset, progressive and refractory periodontal disease and subsequent alveolar bone loss. Pg-fimbriae, in addition to lipopolysaccharide (LPS), are also involved in the pathogenesis of periodontal disease through production of various pro-inflammatory cytokines, which promote the differentiation of osteoclasts (4-6). Pg-fimbriae are peritrichous filamentous appendages,

whereas LPS is a major component of the outer membrane of Pg and is capable of host activation, mediating the adhesion of bacteria to both host cells and a variety of oral substrates and molecules (7).

The polyphenols quercetin, catechin and epicatechin (Figure 1) are major flavonoids widely found in natural plants, and have become a focus of interest because of their various beneficial effects, including strong antioxidant, anti-inflammatory, and anticancer activities (8). In recent years, many reports have indicated that quercetin in particular exerts significant anti-microbial effects on periodontal pathogens *in vitro* (9). Quercetin is capable of interacting with a variety of enzymes including cyclooxygenase (COX), lipoxygenase, phosphodiesterase and tyrosine kinase, and is able to mediate anti-inflammatory processes, mainly by inhibiting the expression of COX1 and -2. Tumor necrosis factor-alpha (TNFα), an essential component of the immune system, is produced by several type cells, especially macrophages. Curcumin and various polyphenols have been shown to exert anti-inflammatory activity by binding directly to pro-inflammatory molecules such as COX1, -2 and TNFα. The antioxidant activity of various combinations of catechin with other polyphenols was previously investigated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and this revealed that some combinations exerted opposite effects, suggesting antagonism (10). However, the antioxidant and anti-inflammatory activity of various combinations of quercetin with catechin or epicatechin at the cellular level has not been sufficiently investigated. It would be of interest to clarify whether such combinations could positively or negatively modulate antioxidant and anti-inflammatory activity in cells because various mixtures of flavonoids are present in natural products.

In the present study, we investigated the inhibitory effects of quercetin, catechin and epicatechin and their combinations on the expression of *Cox2*, *Tnfα* and nitric oxide synthase 2 (*Nos2*) mRNAs stimulated by Pg-fimbriae in a murine macrophage cell line, RAW264.7. We also investigated the anti-DPPH• activity of combinations of quercetin with catechin or epicatechin in order to clarify their radical-scavenging activity resulting from phenolic interactions. Furthermore, in order to clarify the mechanism responsible for the biological activity of quercetin and its related compounds, the relationships between their cytotoxicity or anti-inflammatory activities were investigated in terms of quantum chemical parameters such as σ, χ and Koopman's ionization potential (*IP*) (11).

Materials and Methods

Materials. (+)-Catechin and quercetin were purchased from Tokyo Kasei Co. (Tokyo, Japan). (−)-Epicatechin was obtained from Sigma Aldrich Japan Co. (Tokyo, Japan). The chemical structures of these compounds are shown in Figure 1. Solutions of these compounds were prepared by dissolving each of them in dimethyl sulfoxide, followed by dilution to the indicated concentrations using serum-free RPMI-1640 (Invitrogen Co., Carlsbad, CA, USA) as test samples. Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA), and DPPH from Tokyo Kasei Co. (Tokyo, Japan).

Anti-DPPH activity. The radical-scavenging activities were determined using 0.1 mM DPPH as a free radical. In brief, each of the phenolic compounds was used at different concentrations in ethanol. The decrease in absorbance was determined at 540 nm for 30 min at room temperature. Radical absorbance activity was determined from the dose−response curves as the amount of inhibitor necessary to reduce the initial DPPH• radical concentration. The 50% effective concentration (EC_{50}) for each compound was determined using the dose-response curves. Data are expressed as means of three independent experiments. Statistical analyses were performed using Student's *t*-test.

Phenolic interaction. To evaluate possible interactions, various combinations of two phenolics were studied using the DPPH assay. The procedure employed was similar to that reported previously (10).

Cell culture. The murine macrophage-like cell line RAW264.7, obtained from Dainippon Sumitomo Pharma Biomedical Co. Ltd. (Osaka, Japan), was used. The cells were cultured to a subconfluent state in RPMI-1640 medium supplemented with 10% FBS at 37°C and 5% CO_2 in air, washed, and then incubated overnight in serum-free RPMI-1640. They were then washed again and treated with the test samples.

Cytotoxicity. The relative number of viable cells was determined using a Cell Counting Kit-8 (CCK-8) (Dojindo Co., Kumamoto, Japan) (12). In brief, RAW264.7 cells (3×10^4 per well) were cultured in NUNC 96-well plates (flat-well-type microculture plates) for 48 hours, after which the cells were incubated with test samples for 24 hours. CCK-8 solution was added to each well and then the absorbance was measured at 450 nm with a microplate reader (Biochromatic, Helsinki, Finland). The 50% lethal cytotoxic concentration (LC_{50}) was determined from the dose−response curves. Data are expressed as means of three independent experiments. Statistical analyses were performed using Student's *t*-test.

Preparation of Pg fimbriae. *P. gingivalis* ATCC33277 fimbriae were prepared and purified from cell washings by the method of Yoshimura *et al.* (4). As documented previously, biological activities of purified fimbria-induced were not attributable to LPS contamination of the preparation (5, 6). Viability of the cells after exposure to the fimbriae at the concentrations used was over 90%, as determined using the Cell Counting Kit-8 (Dojindo Co.) (12). The protein content of the fimbriae was measured by the method of Smith *et al.* (13).

Preparation of total RNA and real-time polymerase chain reaction (PCR). The preparation of total RNA and the procedure for real-time PCR have been described previously (14). In brief, RAW264.7 cells in NUNC 96-flat-well-type microculture plates (10^5 cells per
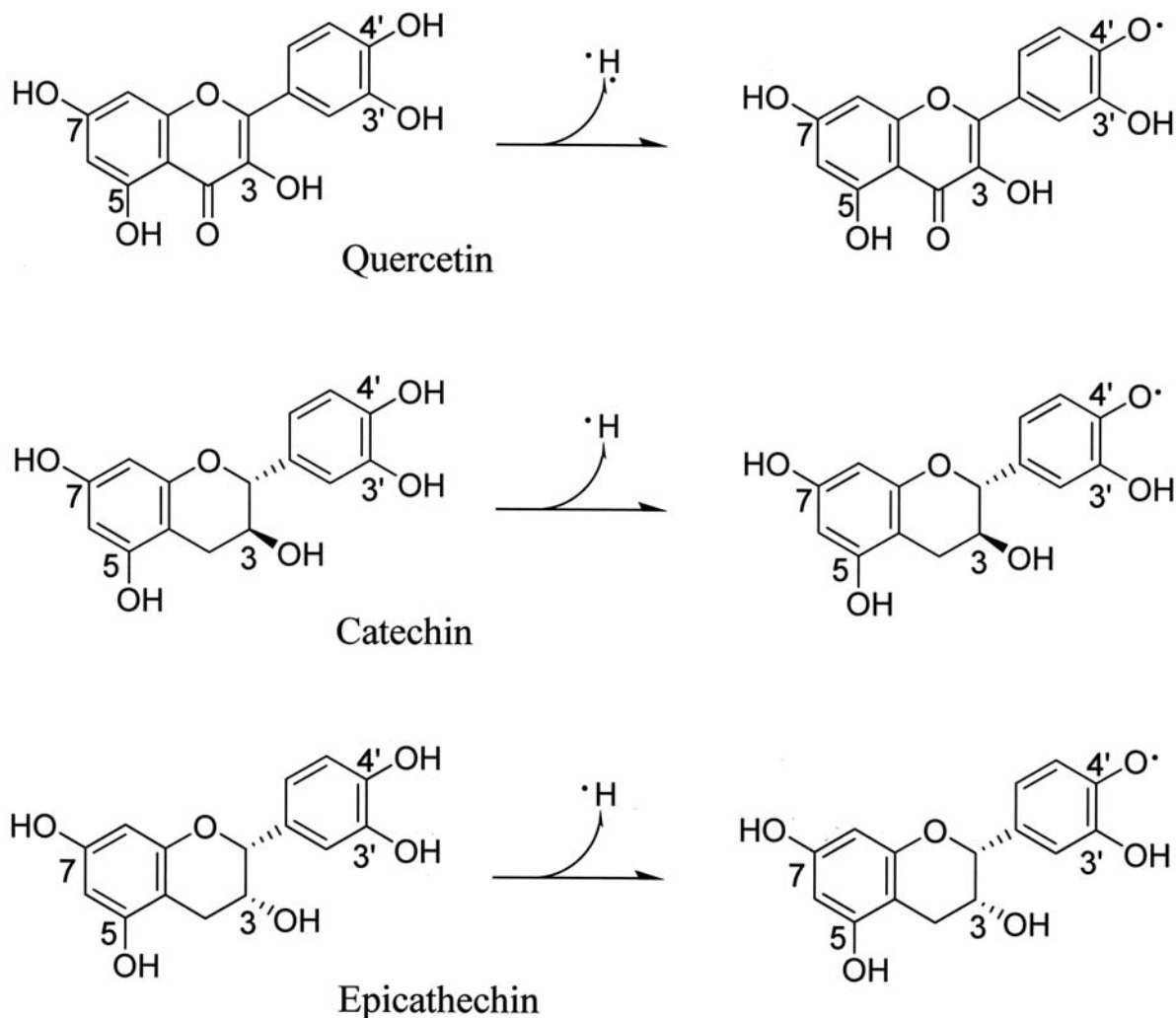
Figure 1. *Chemical structures of catechin, epicatechin and quercetin and their initial oxidation products.*

well) were treated with the test samples. Total RNA was isolated using an RNeasy Plus Micro Kit (Qiagen Japan Co. Ltd., Tokyo, Japan), in accordance with the instruction manual. cDNA was synthesized from total RNA (2 μg) of each sample by random priming using a High Capacity RNA-to-cDNA Kit (Life Technologies Japan, Tokyo, Japan). Reaction mixtures without the reverse transcriptase were also used as a negative control. An aliquot of each cDNA synthesis reaction mixture was diluted and used for real-time PCR quantification. An equal-volume aliquot of each cDNA was mixed, serially diluted, and used as a standard. TaqMan probes/primers for *Cox2, Tnfα, Nos2* and *18S* rRNA and the PCR enzyme mix for real-time PCR were purchased from Life Technologies Japan. Real-time PCR quantification was performed in triplicate using the GeneAmp Sequence Detection System 5700 software (Life Technologies Japan) in accordance with the instruction manual. The relative amount of target was calculated from standard curves generated in each PCR, and quantitative data with a coefficient of variance (CV) of less than 10% were used for

further analyses. Each calculated amount of mRNA was standardized by reference to that for *18S* rRNA. Data are expressed as means of three independent experiments. Statistical analyses were performed using Student's *t*-test.

Computation. The phenolic O-H bond dissociation enthalpy (BDE) for the quercetin-related compounds was calculated as follows: Firstly, the lowest and second-lowest energy conformers of both the phenol derivatives and their phenoxyl radical species were identified as candidates for geometry optimization using the conformer search procedure by Merck Molecular Mechanics force fields calculation. The tentative conformers were then geometrically optimized by *ab initio* molecular orbital calculation at the HF/6-31G* level for the phenols, and with a UHF/6-31G* basis set for the phenoxyl radicals *in vacuo*, to afford the respective energetic minimized structures. The electronic energy was further preceded by single-point calculation involving density functional theory (DFT) using the B3LYP functional at the 6-31G*level: BDE=Hr + Hh − Hp, where
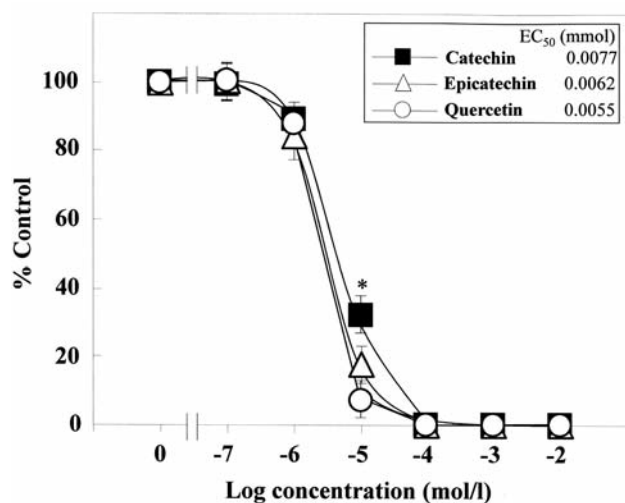
Figure 2. *Radical-scavenging effects of catechin, epicatechin and quercetin. The procedures employed are described in Materials and Methods. The antiradical activity was determined as the amount of inhibitor necessary to reduce the initial diphenylpicrylhydrazyl concentration (0.1 mM) by 50% (EC$_{50}$, mol/l). Values are the means of three separate measurements. The results are presented as the means±standard error (SE). There was a significant difference between quercetin and both catechin and epicatechin up to a concentration of 100 μM. *p<0.01.*
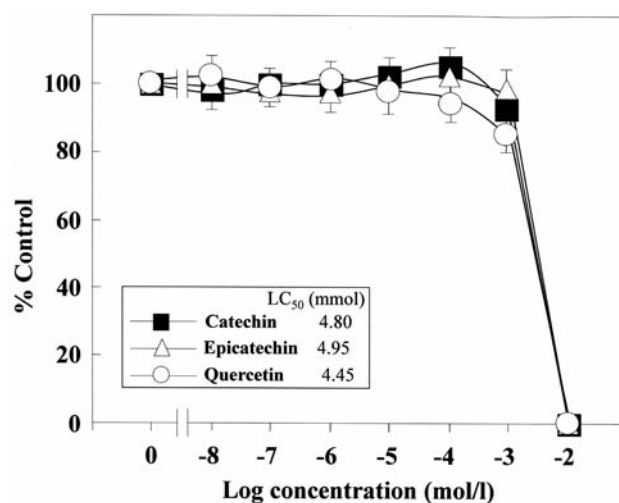

Figure 3. *The cytotoxicity of catechin, epicatechin and quercetin against RAW264.7 cells. The procedures employed are described in Materials and Methods. The results are presented as the means±standard error (SE). There was no significant difference between catechin and epicatechin or quercetin up to a concentration of 10 μM.*

Hr is the enthalpy of the phenoxyl radical generated by hydrogen atom abstraction, Hh is the enthalpy of the hydrogen radical, and Hp is the enthalpy of the parent phenol. The lowest unoccupied molecular orbital (LUMO) energy (E_{LUMO}) and highest occupied molecular orbital (HOMO) energy (E_{HOMO}) were obtained from the ground state equilibrium geometries with the calculated DFT B3LYP 6- 31G* *in vacuo* from 6-31G* initial geometry (14, 15). The absolute value of HOMO energy was adopted as the *IP* value according to Koopman's theorem (11). All calculations were performed using Spartan'10 (Wave Function Inc., Irvine, CA, USA). The chemical hardness (η), softness (σ) and electronegativity (χ) values were calculated as follows:

$$\eta = (E_{LUMO} - E_{HOMO})/2 \qquad \text{(Eq. 1)}$$
$$\sigma = 1/\eta \qquad \text{(Eq. 2)}$$
$$\chi = -(E_{LUMO} + E_{HOMO})/2 \qquad \text{(Eq. 3)}$$

Relationships between cytotoxicity or anti-inflammatory activity and quantum chemical parameters. IBM SPSS version 19 was used for performing correlations.

Results and Discussion

Antioxidant study. The anti-inflammatory activities of flavonoids are largely related to their ability to scavenge oxidative radicals, attenuate nuclear factor kappa B (NfκB) activity, inhibit several genes such as *COX2*, *TNFA* and *NOS2* that are important for regulation of cells, and to prevent viral infection. Therefore, it is important to study the radical-scavenging activity of flavonoids. There have been many previous studies of *in vitro* radical-scavenging activity (10, 16-18). Firstly, using the DPPH assay, we investigated the effects of catechin, epicatechin and quercetin and their combinations, and the results are shown in Figure 2. The EC$_{50}$ values for catechin, epicatechin and quercetin were 7.7, 6.2, and 5.5 μM, respectively. The radical-scavenging activity of quercetin was slightly higher than that of catechin and of epicatechin, with almost equivalent activity against DPPH radicals, being similar to results reported previously (10). To evaluate the antioxidant interaction of these flavonoids, we then examined their combinations using the DPPH assay. As shown in Table I, the calculated absorbance (A), the simple sum of the absorbance of both quercetin and catechin or epicatechin at 2 μM, was significantly smaller than the experimentally determined value (B), *i.e.* a mixture of quercetin with catechin or epicatechin to a total combined concentration of 4 μM. The absorbance ratio of A to B was 1.2-1.3, indicating that the anti-DPPH• activity for A was greater than that for B. As a result, the combination of quercetin with catechin or epicatechin had an opposite effect on antioxidant activity, indicating an antagonistic effect. The radical-scavenging activity of quercetin in combination with catechin or epicatechin was balanced due to their
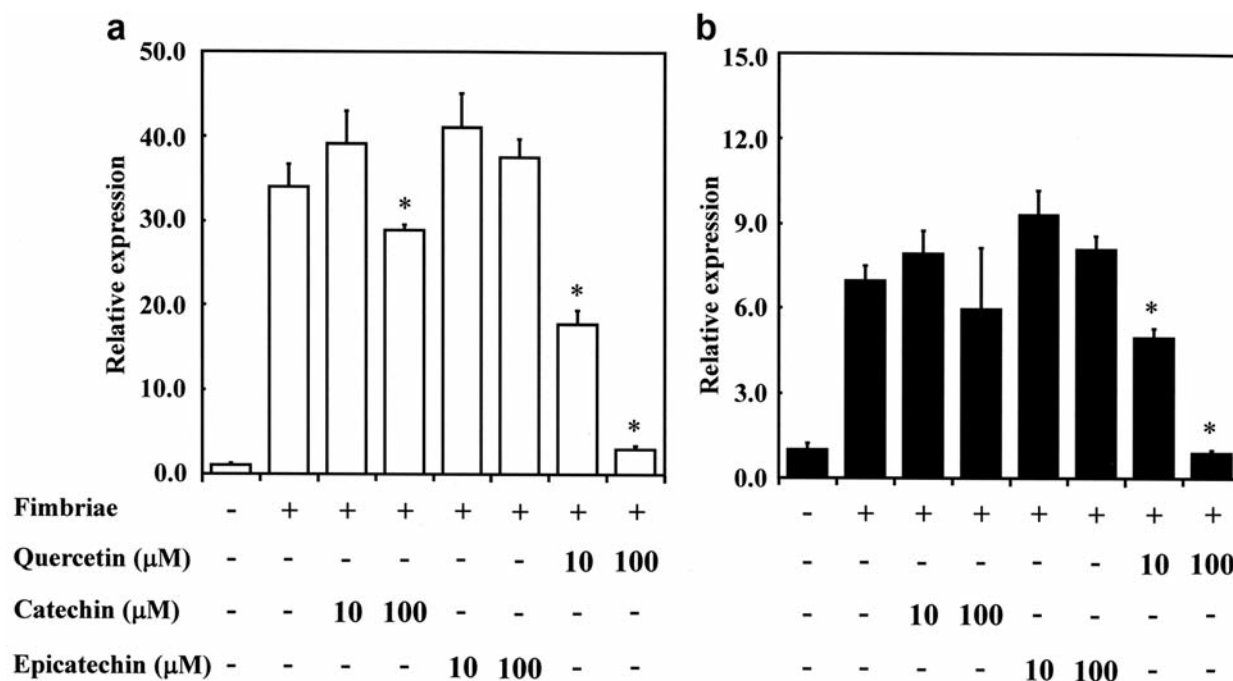
Figure 4. *Inhibitory effects of quercetin, catechin and epicatechin on fimbria-stimulated expression of the cyclooxygenase-2 (Cox2) (a) and tumor necrosis factor-alpha (Tnfa) (b) genes in RAW264.7 cells. The cells were pre-treated for 30 min with the indicated doses of quercetin, catechin and epicatechin. They were then incubated for 3 h with or without fimbriae at 4 μg/ml, and their total RNAs were prepared. Each cDNA was synthesized, and the expression levels of Cox2 and Tnfa mRNAs were determined by real-time PCR and standardized against the expression of 18s rRNA. The results are presented as means±standard error (SE) of three independent experiments. Significant differences between samples for quercetin, catechin and epicatechin were observed for inhibition of Cox2 and Tnfa gene expression. *p<0.01 vs. control group.*

phenolic interaction during oxidation. Lacopini *et al*. reported that the scavenging properties of phenolic combinations of two, three or more catechin-related compounds using DPPH assay were antagonistic (10).

On the other hand, in general, the radical-scavenging activity of phenolic compounds is dependent on the radical species. Some kinetic studies of radical-scavenging activity have been performed on quercetin and related compounds using tocotheroxy- and benzoyl peroxide (BPO)-radical species. Changes in the absorbance of the tocopheroxy radical at 20 nm during reaction of tocopheroxyl radicals with epicatechin or quercetin revealed that the second-order rate constant for epicatechin and quercetin was 1.52×10^2 M^{-1} s^{-1} and 2.98×10^2 M^{-1} s^{-1}, respectively (17). The tocopheroxyl radical-scavenging activity of quercetin is approximately twice that of epicatechin. In our previous study of radical-scavenging activity using the induction period method for polymerization of methyl methacrylate initiated by thermal decomposition of BPO, we found that the inhibition rate constant (kinh) for catechin and quercetin was 1.05×10^3 M^{-1} s^{-1} and 1.60×10^3 M^{-1} s^{-1}, respectively, the kinh value for quercetin being about 1.6 times higher than that for catechin (18). The radical-scavenging activity of combinations of quercetin and epicatechin using the

Table I. *Antioxidant interactions on diphenylpicrylhydrazyl (DPPH•) free radical-scavenging activity for quercetin, epicatechin and catechin and their combinations[a].*

Treatment	A	B	A-B	B/A	Effect
Quercetin		15.0±0.7			
Epicatechin		15.1±0.2			
Catechin		6.4±0.2			
Quercetin+epicatechin	30.1±0.9	35.0±0.8	4.9±0.1	1.16*	Antagonism
Quercetin+catechin	21.4±0.9	27.0±0.3	5.6±0.6	1.26*	Antagonism
Catechin+epicatechin	21.5±0.4	28.0±0.5	6.5±0.1	1.3*	Antagonism

[a]The reduction rate was expressed as [(A_{540} of 0.1 mM DPPH−A_{540} of sample)/A_{540} of 0.1 mM DPPH] ×100. Values are the mean±SD (n=4). A: Calculated absorbance (%), simple sum of quercetin+epicatechin (or catechin), or catechin and epicatechin; B: experimentally recorded absorbance (%) for the indicated phenols and their mixtures. A *vs*. B, *p<0.01.

induction period method also indicated an antagonistic effect (16). Our findings using the DPPH• assay were consistent with those reported above. Combinations of quercetin with catechin or epicatechin were concluded to negatively affect the antioxidant activity.
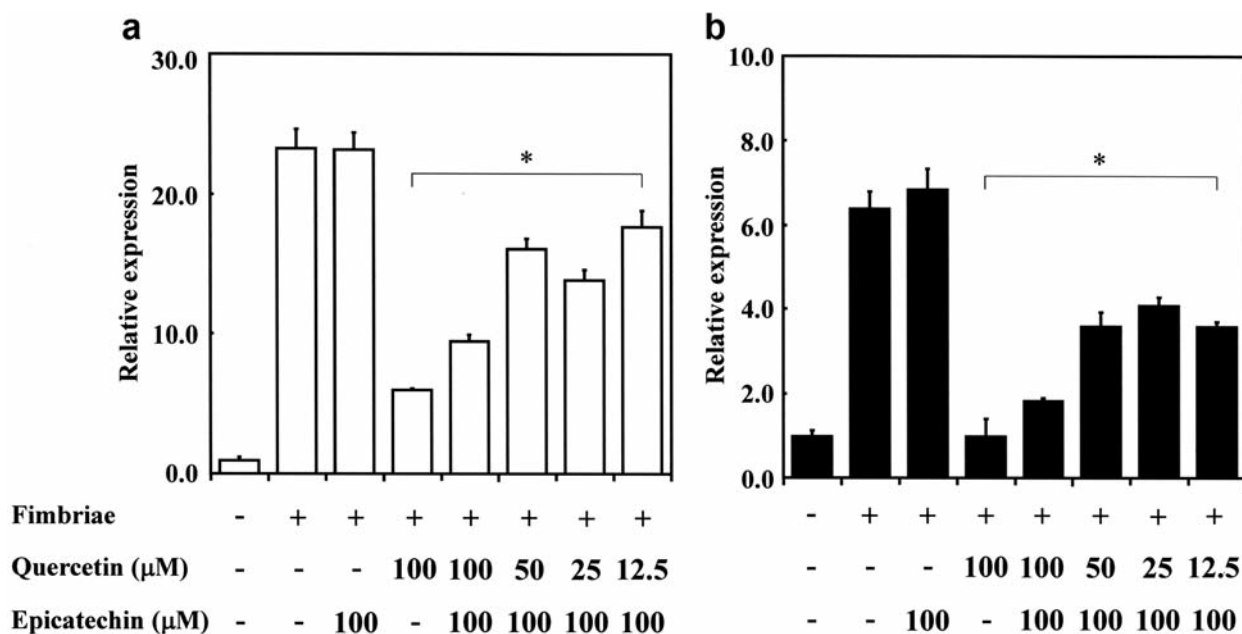
Figure 5. *Inhibitory effects of quercetin and epicatechin on fimbria-stimulated expression of the cyclooxygenase-2 (Cox2) (a) and tumor necrosis factor-alpha (Tnfa) (b) genes in RAW264.7 cells. The cells were pre-treated for 30 min with the indicated doses of quercetin and epicatechin. They were then incubated for 3 h with or without fimbriae at 4 μg/ml, and their total RNA were then prepared. Each cDNA was synthesized, and the expression levels of Cox2 and Tnfa mRNAs were determined by real-time PCR and standardized against the expression of 18s rRNA. The results are presented as means±standard error (SE) of three independent experiments. Significant differences between samples for quercetin and epicatechin were observed for inhibition of Cox2 and Tnfa gene expression. *p<0.01 vs. control group.*

Cox2/Tnfa/Nos2 studies. Peroxynitrites and hydroxy radicals are derived from lipid peroxidation, disruption of cellular structures, inactivation of enzymes and ion channels via protein oxidation and nitration, and DNA damage (19). All these actions are associated with the onset and persistence of inflammation. Radical scavengers such as quercetin may prevent inflammation derived from LPS and bacterial fimbriae. Firstly, therefore, we investigated the cytotoxicity (LC_{50}) of three flavonoids against RAW264.7 cells. The LC_{50} values for catechin, epicatechin and quercetin were 4,800, 4,950 and 4,450 μM, respectively (Figure 3). The cytotoxicity of quercetin was slightly higher than that of catechin and of epicatechin, but the difference in cytotoxicity between the compounds was very small.

Next, using RAW264.7 cells stimulated with Pg-fimbriae at non-cytotoxic concentrations, we investigated the expression of *Cox2* and *Tnfα* mRNA after treatment with quercetin, catechin and epicatechin and their combinations. The results are also shown in Figures 4, and 5, respectively. For expression of *Cox2* mRNA, the 50% effective concentration ($Cox2IC_{50}$) of quercetin was approximately 10 μM, whereas that for both catechin and epicatechin was approximately 500 μM (Figure 6b). The anti-inflammatory activity of quercetin was approximately one-order of magnitude greater than that of catechin or epicatechin. It was considered that the anti-inflammatory activity of quercetin was potent, whereas that of catechin and epicatechin, steric isomers, were considerably weak. As shown in Figures 4b and 5b, the inhibitory activity of quercetin, catechin and epicatechin on *Tnfα* expression was almost the same as that on *Cox2* expression. The inhibitory effect of the quercetin/epicatechin combination on *Cox2* or *Tnfa* mRNA expression was also investigated, and results are shown in Figures 5a and b, respectively. The inhibitory effect of quercetin on both *Cox2* and *Tnfa* expression was not enhanced by the addition of epicatechin. We incubated different concentrations of quercetin in the presence of 100 μM epicatechin and investigated its anti-inflammatory activity at molar ratios of 1, 2, 4 and 8. We found that the anti-inflammatory activity predominantly induced by quercetin and was not modulated by the addition of epicatechin. Addition of epicatechin to quercetin negatively affected the anti-inflammatory activity. This may be related to the antagonistic effect of the combination between quercetin and epicatechin on the radical-scavenging activity (Table I). The combination of quercetin with epicatechin was considered to have an opposite effect on antioxidant and anti-inflammatory activity.
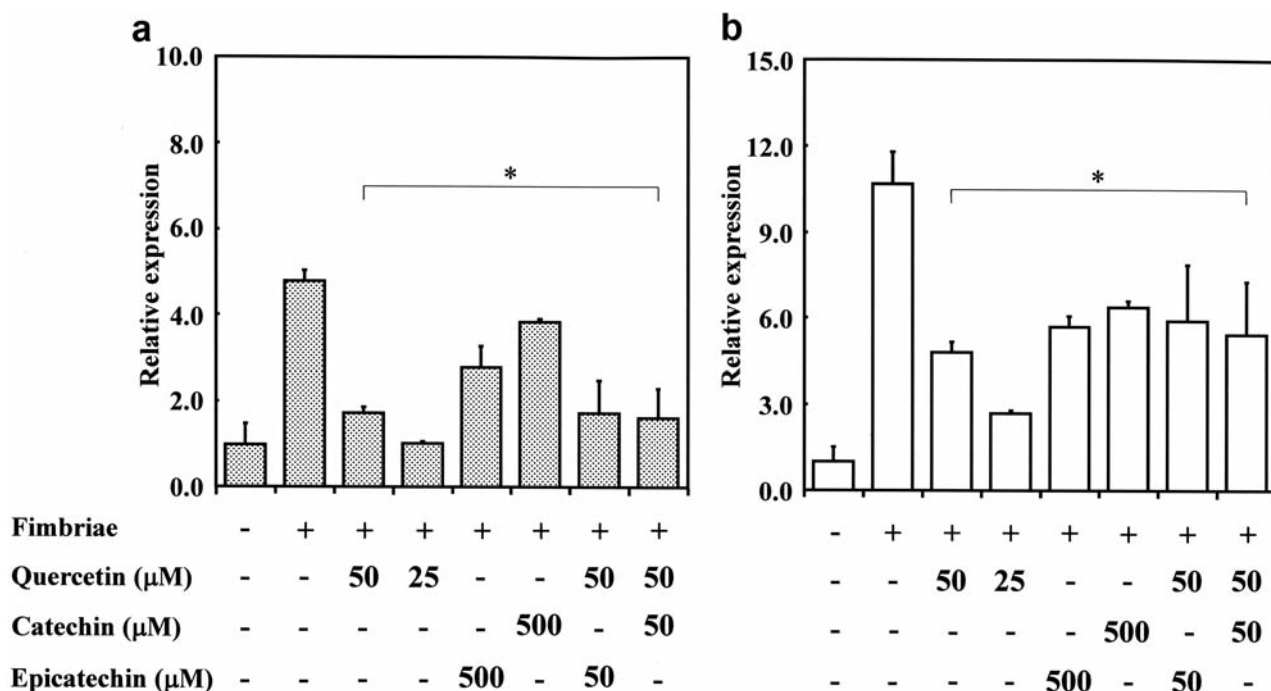
Figure 6. *Inhibitory effects of quercetin, catechin and epicatechin on fimbria-stimulated expression of the nitric oxide synthase-2 (Nos2) (a) and cyclooxygenase-2 (Cox2) (b) genes in RAW264.7 cells. The cells were pre-treated for 30 min with the indicated doses of quercetin, catechin and epicatechin. They were then incubated for 3 h with or without fimbriae at 4 µg/ml, and their total RNAs were prepared. Each cDNA was synthesized, and the expression levels of Nos2 and Cox2 mRNAs were determined by real-time PCR and standardized against the expression of 18s rRNA. The results are presented as means±standard error (SE) of three independent experiments. Significant differences between samples for quercetin, catechin and epicatechin were observed for inhibition of Nos2 and Cox2 gene expression. *p<0.01 vs. control group.*

Mu *et al*. reported that quercetin pre-treatment significantly inhibited NO production in LPS-stimulated RAW264.7 cells, the expression of *Nos2* being markedly down-regulated *via* inhibition of Nf-κB (20). Therefore, we further investigated the inhibitory effects of catechin, epicatechin, quercetin and their combinations on Pg-fimbria-stimulated expression of *Nos2* and *Cox2* mRNA in RAW264.7 cells using real-time PCR. Figure 6 shows that quercetin at concentrations of both 25 µM and 50 µM strongly inhibited the fimbria-induced expression of *Nos2*, whereas catechin and epicatechin showed slight inhibition even at a relatively high concentration of 500 µM. *Nos2* gene expression for combinations of quercetin and catechin or epicatechin at 50 µM was also similar to that for quercetin alone at the same concentration. The inhibitory effect of combinations of quercetin with catechin or epicatechin on *Cox2* mRNA expression was also similar to that on *Nos2* mRNA expression (Figure 6b). These findings appear to be consistent with the antagonistic effect of the radical-scavenging activity of phenolic combinations determined using the anti-DPPH• assay (Table I). The *Nos2* mRNA expression after treatment with flavonoids was closely related to their antioxidant activity.

To date, most researchers have focused on the antioxidant and anti-inflammatory activity of quercetin-related compounds, however the mechanism and structural requirements for their antioxidant activity are not fully understood at the cellular level. One hypothesis to explain the inhibitory effect of quercetin on Pg-fimbria-stimulated expression of *Nos2* and *Cox2* mRNAs in RAW264.7 cells has stressed the importance of a catechol-like structure (the presence of 3' and 4' hydroxy groups in the B ring) and the C2-C3 double bond in the C ring (Figure 1) (21). However, catechin and epicatechin lack a C2-C3 double bond in the C ring. The inhibitory effect of quercetin on expression of *Nos2* mRNA may be attributable to the presence of the C2-C3 double bond in the C ring. It was previously reported that the position, number and substitution of the hydroxy group in the B ring, and saturation of the C2-C3 double bond are important factors affecting the inhibition of kinases by flavonoids. The minimum structural requirement for kinase-binding activity was previously reported to be the presence of a C2-C3 double bond and polar groups on the C ring (22). We previously investigated the anti-inflammatory activity of curcumin and tetrahydrocurcumin and found that the activity of the former was markedly higher than that of the latter,

Table II. *Cytotoxicity (50% cytotoxic concentration, LC$_{50}$) and the ability to induce a 50% decrease in Porphyromonas gingivalis-fimbria-stimulated cyclo-oxygenase-2 (Cox2) mRNA expression (^{Cox2}IC$_{50}$) in RAW264.7 cells for flavonoids, and their quantum chemical parameters.*

	Quercetin	Catechin	Epicatechin	Resveratrol[a]
Cytotoxicity (LC$_{50}$ mM)	4.45	4.80	4.95	0.45
Cox2IC$_{50}$ (μM)	10	500	500	<50, ca 30
E$_{HOMO}$ (eV)	−5.481 (−5.69)[b]	−5.664 (−5.63)[c]	−5.683 (−5.69)[c]	−5.26
E$_{LUMO}$ (eV)	−1.843 (−2.30)[b]	−0.188 (−0.38)[c]	0.01 (−0.44)[c]	−1.21
IP$_{koopman}$ (eV)	5.481	5.664	5.683	5.26
|E$_{HOMO}$-E$_{LUMO}$| (eV)	3.638	5.456	5.694	4.05
Hardness (η) (eV)	1.819	2.728	2.847	2.03
Softness (σ) (eV)	0.550	0.367	0.351	0.49
Electronegativity (χ) (eV)	3.662	2.916	2.836	3.23

[a]Ref. (27)., [b]Ref. (24)., [c]Ref. (25). Note: The lowest unoccupied molecular orbital (LUMO) energy (E$_{LUMO}$), highest occupied molecular orbital (HOMO) energy (E$_{HOMO}$), ionization potential (IP)=|E$_{HOMO}$|, η=(E$_{LUMO}$−E$_{HOMO}$)/2, σ=1/η, χ=−(E$_{LUMO}$ + E$_{HOMO}$)/2, calculations at DFT-B3LYP/6-31G* level.

possibly due to the fact that curcumin has phenol rings connected by two α, β-unsaturated carbonyl groups (double bond), whereas tetrahydrocurcumin lacks the unsaturated carbonyl group (23). Interestingly, phenolic interactions between quercetin and epicatechin or catechin negatively affected the anti-inflammatory activity and therefore the synergistic anti-inflammatory activities of quercetin in combination with other flavonoids are questionable. However, a more detailed study is necessary to substantiate this hypothesis.

BDE, IP, σ and χ. Quantum chemical calculation might provide a clearer insight into the molecular mechanisms of radical-scavenging and anti-inflammatory activity of these phenolic compounds. The HOMO and LUMO energy for three flavonoids and resveratrol were calculated, and the results are shown in Table II. The HOMO and LUMO values for quercetin, catechin and epicatechin were similar to those reported previously (24, 25). Since BDE and IP for flavonoid antioxidants can represent radical-scavenging to a large extent, we calculated the BDE and IP values for flavonoids at the DFT-B3LYP/6-31G* level. The BDE values for flavonoids (see Figure 1) are shown in Table III. The BDE of 4'-OH (kcal/mol), the initial active site in the B-ring for flavonoids, declined in the order catechin (75.9) > epicatechin (75.3) > quercetin (75.1). By contrast, the corresponding BDE for resveratrol at 3'-OH (the initial active site in the B-ring) was 80.77 kcal/mol, being about 5 kcal/mol higher than that for flavonoids. The IP value is the most important energy factor for evaluation of scavenging ability (11). A relatively high *IP* value decreases the rate of electron transfer between a phenolic antioxidant and oxygen, and therefore phenolic compounds with a higher *IP* value have lower pro-oxidative potency, and *vice versa* (26). The *IP* value (eV) declined in the order catechin ≈ epicatechin (5.7) > quercetin (5.4) > resveratrol (5.3) (Table II). It has been

shown experimentally that the anti-DPPH• activity of quercetin, catechin or epicatechin with a catechol-like substitution on the B ring is about 8 times greater than that of resveratrol with a mono phenol on the B ring (EC$_{50}$ 45 μM) (27). In general, low BDE and *IP* values indicate a high antioxidant activity, but an extremely low IP value may result in a change from an antioxidant to a prooxidant character. The low anti-DPPH• activity of resveratrol may be related to its high BDE value. The cytotoxicity of resveratrol was also one order of magnitude greater than that of quercetin, catechin or epicatechin (Table II), possibly due to the small *IP* value of this compound. Phenolic compounds with a small *IP* value tend to act as pro-oxidants in the presence of reactive oxygen species and increase the cytotoxic potential of phenolics.

Molecules with a relatively small HOMO-LUMO energy gap (|E$_{HOMO}$ − E$_{LUMO}$|) are generally reactive, while those with a relatively large value are generally not reactive (15). As shown in Table II, the softness (σ) value (σ=1/η; *i.e.* the inverse value of hardness) of quercetin was highest among the three flavonoids tested, indicating that quercetin is more reactive than catechin or epicatechin. In contrast, the χ value represents the chemical potential of the electrophile. According to the Frontier Orbital Theory, adduct formation occurs when a soft nucleophile donates its highest-energy electrons to the empty lowest-energy orbital of a soft electrophile. Hence, the most relevant frontier orbital for electrophiles is the LUMO, whereas the HOMO is most important for nucleophiles. The σ value is defined as the ease with which electron re-distribution can take place during covalent bonding, and thus the softer the electrophile (*i.e.* the more negative the E$_{LUMO}$ value and the higher the σ value), the more readily it will form an adduct by accepting outer-shell electrons from a soft nucleophile such as a sulfur atom (1). Investigation of the relationship between anti-inflammatory activity and quantum chemical parameters for

Table III. *Calculated phenolic O-H bond dissociation enthalpy (kcal mol^{-1}) for polyphenols.*

Radial position[a]	Catechin	Epicatechin	Quercetin	Resveratrol
3	104.79	104.01	82.61	–
5	83.35	83.68	112.12	–
7	86.58	86.96	107.31	–
3'	76.10	75.35	77.71	–
4'	75.87	75.26	75.05	80.77

[a]See Figure 1.

Table IV. *Correlation analysis of the relationship between the cytotoxicity (LC$_{50}$) or Cox2-inhibitory activity (Cox2IC$_{50}$) for quercetin, epicatechin, catechin and resveratrol and their quantum chemical parameters.*

	LC$_{50}$	Log Cox2IC$_{50}$
LC$_{50}$	1	0.462
Log ^{Cox2}IC$_{50}$	0.462	1
IP	0.926	0.762
σ	−0.437	−0.997**
χ	−0.217	−0.961*

IP: Ionization potential; σ: softness; χ: electronegativity. *$p<0.05$, **$p<0.01$.

resveratrol-related compounds has recently revealed that resveratrol, that has potent anti-inflammatory activity, has a large χ value, but also a small η value, *i.e.* a large σ value (27). Here we investigated the correlation between cytotoxicity or anti-inflammatory activity, in terms of inhibition of *Cox2* mRNA expression, and the σ, *IP* and χ values for four polyphenols: quercetin, catechin, epicatechin and resveratrol (Table II). The results are shown in Table IV. There were no significant relationships of σ, *IP* or χ with cytotoxicity. By contrast, the logarithmic value of anti-inflammatory activity (Cox2IC$_{50}$) for these polyphenols had a significant linear relationship to the σ and to the χ values, suggesting that Cox2IC$_{50}$ may be dependent on σ or χ. These data clearly demonstrate that the potent inhibitory effect of quercetin on Pg-fimbria-induced *Cox2* expression in RAW264.7 cells may be related to its high σ or χ value, although some other mechanism may also contribute to the net effect.

It has been considered that polyphenols, capable of interacting with and modulating the activity of a variety of enzymes including COX, lipoxygenase, phosphodiesterase and tyrosine kinase, are the most potent non-steroidal anti-inflammatory drug-like compounds. It has been proposed that quercetin has an inhibitory effect on protein tyrosine kinase (28) and inhibits the interaction of carcinogens with DNA (29). Quercetin supplementation has also been reported to up-regulate the mRNA and protein levels of the intercellular antioxidant enzyme paraoxonase 2 (*Pon2*) in cultured RAW264.7 cells (30). The beneficial effects of quercetin in biological systems may be associated with its interaction with enzymes, as it possesses antioxidant activities that prevent free radical damage to biological molecules. Quercetin has high σ and χ values, allowing it to bind to the active site cavity with a favorable ligand–protein molecular interaction. On the other hand, oxidized flavonoids readily and specifically adduct thiol groups, and may disrupt vital cellular compounds containing such groups (31). When quercetin is incubated in the presence of glutathione, a quinoid derivative is produced through hydroperoxidase action (32). Quinoid derivatives exert adverse effects in biological systems. Since quercetin possesses high σ and χ values, it may exert stronger pro-inflammatory activity

and toxic effects in the presence of ROS. A quinone derived through the oxidation of quercetin would preferentially attack macromolecules (DNA and enzymes) in biological systems due to the reactivity of unsaturated compounds with nucleophilic additions (33, 34). It is likely that quercetin can scavenge many intercellular free radicals, and the various metabolites of quercetin, including quercetin-derived radicals, are probably involved in toxicity. More detailed studies of the anti-inflammatory and antioxidant actions of quercetin and related compounds will be required in order to understand their true potential as preventive agents against chronic infections and various oral diseases, including cancer.

Acknowledgements

References

1 Bascones-Martínez A, González-Febles J and Sanz-Esporrín J: Diabetes and periodontal disease. Review of the literature. Am J Dent *27*: 63-67, 2014.

2 Mattila KJ, Pussinen PJ and Paju S: Dental infections and cardiovascular diseases: a review. J Periodontol *76(Suppl)*: 2085-2088, 2005.

3 Offenbacher S, Katz V, Fertik G, Collins J, Boyd D, Maynor G, McKaig R, Beck J: Periodontal infection as a possible risk factor for preterm low birth weight. J Periodontol *67(Suppl)*: 1103-1113, 1996.

4 Yoshimura F, Takahashi K, Nodasaka Y and Suzuki T: Purification and characterization of a novel type of fimbriae from the oral anaerobe Bacteroides gingivalis. J Bacteriol *160*: 949-954, 1984.

5 Hanazawa S, Murakami Y, Hirose K, Amano S, Ohmori Y, Higuchi H and Kitano S: Bacteroides (Porphyromonas) gingivalis fimbriae activating mouse peritoneal macrophages and gene expression and production of interleukin-1. Infect Immun *59*: 1972-1977, 1991.

6 Hanazawa S, Kawata Y, Murakami Y, Naganuma K, Amano S, Miyata Y and Kitano S: *Porphyromonas gingivalis* fimbria-stimulated bone resorption *in vitro* is inhibited by a tyrosine kinase inhibitor. Infect Immun *63*: 2374-2377, 1995.

7 Nakagawa I, Amano A, Kimura RK, Nakamura T, Kawabata S and Hamada S: Distribution and molecular characterization of *Porphyromonas gingivalis* carrying a new type of fimA gene. J Clin Microbiol *38*: 1909-1914, 2000.

8 Wang S, Moustaid-Moussa N, Chen L, Mo H, Shastri A, Su R, Bapat P, Kwun I and Shen CL: Novel insights of dietary polyphenols and obesity. J Nutr Biochem *25*: 1-18, 2014.

9 Geoghegan F, Wong RW and Rabie AB: Inhibitory effect of quercetin on periodontal pathogens *in vitro*. Phytother Res *24*: 817-820, 2010.

10 Lacopini P, Baldi M, Storchi P and Sebastiani L: Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: Content, *in vitro* antioxidant activity and interactions J Food Compos Anal 21: 589– 598, 2008.

11 Koopmans T: The distribution of wave function and characteristic value among the individual electrons of an atom. Physica 1: 104-113, 1934.

12 Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y and Ueno K: A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta 44: 1299-1305, 1997.

13 Smith PM, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC: Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76-85, 1985.

14 Murakami Y, Kawata A, Seki Y, Koh T, Yuhara K, Maruyama T, Machino M, Ito S, Kadoma Y and Fujisawa S:Comparative inhibitory effects of magnolol, honokiol, eugenol and bis-eugenol on cyclooxygenase-2 expression and nuclear factor kappa B activation in RAW264.7 macrophage-like cells stimulated with fimbriae of *Porphyromonas gingivalis*. In Vivo 26: 941-950, 2012.

15 Lopachin RM, Gavin T, Decaprio A and Barber DS: Application of the Hard and Soft, Acids and Bases (HSAB) theory to toxicant–target interactions. Chem Res Toxicol 25: 239-251, 2012.

16 Kadoma Y and Fujisawa S: Radical-scavenging activity of dietary phytophenols in combination with co-antioxidants using the induction period method. Molecules 16: 10457-10470, 2011.

17 Mukai K, Mitani S, Ohara K and Nagaoka S: Structure–activity relationship of the tocopherol-regeneration reaction by catechins. Free Radic Biol Med 38: 1243-1256, 2005.

18 Fujisawa S, Ishihara M and Kadoma Y: Kinetic evaluation of the reactivity of flavonoids as radical scavengers. SAR QSAR Environ Res 13: 617-627, 2002.

19 Virág L, Szabó E, Gergely P and Szabó C: Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. Toxicol Lett 140-141: 113-124, 2003.

20 Mu MM, Chakravortty D, Sugiyama T, Koide N, Takahashi K, Mori I, Yoshida T and Yokochi T: The inhibitory action of quercetin on lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophage cells. J Endotoxin Res 7:431-438, 2001.

21 Ferrali M, Signorini C, Caciotti B, Sugherini L, Ciccoli L, Giachetti D and Comporti M: Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron-chelating activity. FEBS Lett 416: 123-129, 1997.

22 Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Rémésy C, Chap H and Payrastre B: Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem Pharmacol 53: 1649-1657, 1997.

23 Murakami Y, Ishii H, Takada N, Tanaka S, Machino M, Ito S and Fujisawa S: Comparative anti-inflammatory activities of curcumin and tetrahydrocurcumin based on the phenolic O-H bond dissociation enthalpy, ionization potential and quantum chemical descriptor. Anticancer Res 28: 699-707, 2008.

24 Mendoza-Wilson AM, Glossman-Mitnik D: CHIH-DFT study of the electronic properties and chemical reactivity of quercetin. Theochem 716: 67-72, 2005.

25 Mendoza-Wilson AM, Glossman-Mitnik D: Theoretical study of the molecular properties and chemical reactivities of (+)-catechin and (−)-epicatechin related their antioxidant activity. Theochem 761: 97-106, 2006.

26 Zhang HY, Sun YM and Wang XL: Substituent effects on O-H bond dissociation enthalpies and ionization potentials of catechols: a DFT study and its implications in the rational design of phenolic antioxidants and elucidation of structure–activity relationships for flavonoid antioxidants. Chemistry 20: 502-508, 2003.

27 Murakami Y, Kawata A, Ito S, Katayama T and Fujisawa S: The radical scavenging activity and cytotoxicity of resveratrol, orcinol and 4-allylphenol and their inhibitory effects on Cox2 gene expression and Nfkb activation in RAW264.7 cells stimulated with *Porphyromonas gingivalis*-fimbriae. In Vivo 29: 341-350, 2015.

28 Srivastava AK: Inhibition of phosphorylase kinase, and tyrosine protein kinase activities by quercetin. Biochem Biophys Res Commun 131: 1-5, 1985.

29 Nguyen TT, Tran E, Nguyen TH, Do PT, Huynh TH and Huynh H: The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. Carcinogenesis 25: 647-659, 2004.

30 Boesch-Saadatmandi C, Pospissil RT, Graeser AC, Canali R, Boomgaarden I, Doering F, Wolffram S, Egert S, Mueller MJ and Rimbach G: Effect of quercetin on paraoxonase 2 levels in RAW264.7 macrophages and in human monocytes–role of quercetin metabolism. Int J Mol Sci 10: 4168-4177, 2009.

31 Boots AW, Haenen GR, den Hartog GJ and Bast A: Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. Biochim Biophys Acta 1583: 279-284, 2002.

32 Pinto Mdel C and Macias P: Oxidation of dietary polyphenolics by hydroperoxidase activity of lipoxygenase. J Agric Food Chem 53: 9225-9230, 2005.

33 Enoch SJ, Cronin MT, Schultz TW and Madden JC: Quantitative and mechanistic read across for predicting the skin sensitization potential of alkenes acting via Michael addition. Chem Res Toxicol 21: 513-520, 2008.

34 Lemmens KJ, Vrolijk MF, Bouwman FG, van der Vijgh WJ, Bast A and Haenen GR: The minor structural difference between the antioxidants quercetin and 4'O-methylquercetin has a major impact on their selective thiol toxicity. Int J Mol Sci 15: 7475-7484, 2014.