Research Article

Correlation between antiangiogenic activity and antioxidant activity of various components from propolis

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Propolis possesses various physiological activities. In this study, we examined the antiangiogenic and antioxidant activities of various components from propolis: acacetin, apigenin, artepillin C, caffeic acid phenethyl ester, chrysin, *p*-coumaric acid, galangin, kaempferol, pinocembrin, and quercetin. The effects of these components were tested on *in vitro* models of angiogenesis, tube formation and growth of human umbilical vein endothelial cells (HUVECs). Furthermore, these components were evaluated for their antioxidant activities by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging and ferric reducing/antioxidant power (FRAP) assays. Two propolis components, caffeic acid phenethyl ester, and quercetin, possessed strong inhibitory effects on tube formation and on endothelial cell proliferation and, coincidentally, showed strong antioxidant activity. Artepillin C, galangin, and kaempferol also possessed strong antiangiogenic and antioxidant activities to a slightly less degree. In contrast, acacetin, apigenin, and pinocembrin possessed a considerable degree of antiangiogenic activities, although they showed very low antioxidant activities. From these results, we propose that components from propolis such as artepillin C, caffeic acid phenethyl ester, galangin, kaempferol, and quercetin might represent a new class of dietary-derived antioxidative compounds with antiangiogenic activities. These propolis components may have the potential to be developed into pharmaceutical drugs for the treatment of angiogenesis-dependent human diseases such as tumors.

Keywords: Angiogenesis / Antioxidant / Human umbilical vein endothelial cells / Propolis / Tube formation

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1 Introduction

Propolis, a natural substance collected by honeybees from buds and exudates of certain trees and plants, is thought to be used in the beehives as a protective barrier against their enemies. It has been used in folk medicine from ancient times for its pharmaceutical properties and has been extensively studied in many countries [1–3]. Propolis contains a variety of chemical compounds such as polyphenols (flavonoids, phenolic acids, and their esters), terpenoids, steroids, and amino acids [4–8]. Recently, it has been reported to possess various biological activities such as antibacterial [9, 10], antiviral [9, 11], anti-inflammatory [12, 13], anticancer [14–18], and antifungal [9, 19] properties. For such reasons, propolis is used in foods and beverages to improve health and to prevent diseases such as inflammation, diabetes, heart disease, and cancer [3, 20].

Angiogenesis, or new blood vessel growth, is defined as a process in which a network of new blood vessels emerges from preexisting vessels [21]. It has been shown that exponential tumor growth over a few mm^3 in size is dependent on the recruitment of its own nutrition and oxygen supply by angiogeneis [22]. Since then many investigators have pursued studies for preventing or delaying cancer growth, or even completely eliminating cancer from a patient's body, by suppressing such neovascularization [23]. Food

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Abbreviations: BHT, butylated hydroxytoluene; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **FBS,** fetal bovine serum; **FRAP,** ferric reducing/antioxidant power; **HUVEC,** human umbilical vein endothelial cell; **ROS,** reactive oxygen species; **VE,** α-tocopherol

factors capable of inhibiting angiogenesis, if found, would be useful to stop the progression of small cancers [24].

In our previous paper, we reported the antiangiogenic effects of Brazilian propolis and its major component artepillin C *in vitro* and *in vivo* [25]. We and other groups have been extensively studying antioxidant activities of propolis and its components [6–8, 26–32]. It has been shown that the disease-preventing activity, such as antiangiogenic effect, of propolis may partly be attributed to antioxidant activity of its components [18, 33]. Further evaluation of their biological activities and elucidation of the mechanisms of their actions may provide substantial clues for the development of new drug candidates from propolis components.

In this study, we investigated antiangiogenic and antioxidant activities of various components from propolis: acacetin, apigenin, artepillin C, caffeic acid phenethyl ester, chrysin, *p*-coumaric acid, galangin, kaempferol, pinocembrin, and quercetin. We analyzed the effects on angiogenesis *in vitro* through inhibition of tube formation and endothelial cell proliferation by various components from propolis. Furthermore, we used two assay systems for evaluating the *in vitro* antioxidant activity of various components from propolis: the free radical-scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the ferric reducing/antioxidant power (FRAP) assay.

2 Materials and methods

2.1 Materials

Acacetin (a), apigenin (b), chrysin (e), galangin (g), kaempferol (h), pinocembrin (i), and quercetin (j) were purchased from Funakoshi (Tokyo, Japan). Artepillin C (c) was synthesized according to the method of our previous report [34]. Caffeic acid phenethyl ester (d) and DPPH were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Medium 199, *p*-coumaric acid (f), α-tocopherol (VE), and all other chemicals were purchased from Sigma (St. Louis, MO, USA) unless noted otherwise. Medium MCDB-104 was a product of Nihon Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Moregate (Brisbane, Australia). Cellgen was obtained from Koken (Tokyo, Japan). Epidermal growth factor (EGF) was purchased from BD Biosciences (Bedford, MA, USA). Endothelial cell growth factor (ECGF) was purified according to our previous report [35]. Human basic fibroblast growth factor (bFGF) (recombinant) was purchased from Austral Biologicals (San Ramon, CA, USA). Butylated hydroxytoluene (BHT) was purchased from Kanto Chemicals (Tokyo, Japan).

2.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord and were grown in HUVEC growth medium (MCDB-104 medium supplemented with 10 ng/mL EGF, 100 μg/mL heparin, 100 ng/mL ECGF, and 10% FBS) as previously reported [35]. Incubation was carried out at 37°C under a humidified 95–5% v/v mixture of air and CO_2. The cells were seeded on plates coated with 0.1% gelatin and allowed to grow to subconfluence before experimental treatment. Cells of passages 5–8, equivalent to population doubling levels of 20.8–30.8, in the actively growing condition were used for experiments.

2.3 Tube formation assay

HUVECs were induced to form capillary tube-like structures in type I collagen gel (Cellgen) as previously described with slight modifications [25, 35]. Aliquots (200 μL) of collagen solution (0.21% in M 199) were poured into the wells of 24-multiwell culture plates, and the plates were incubated at 37°C for 30 min to solidify gels. HUVECs (6.0×10^4 cells/cm^2) in MCDB-104 with 0.5% FBS were seeded onto the collagen gels and left at 37°C for 1 h in a 5% CO_2 incubator for attachment. After removing the medium, 150 μL aliquots of the collagen solution were overlaid and subjected to gelation as described above. Subsequently, 650 μL aliquots of MCDB-104 with 0.5% FBS supplemented with 10 ng/mL bFGF, 8 nM PMA, and 25 μg/mL ascorbic acid with various components of propolis (3.13, 12.5, and 50 μg/mL) were added to the wells and incubated for 36 h. The resulting web-like capillary structure was viewed with a microscope under 100 × magnification and captured with an Olympus C 4040-ZOOM digital camera.

2.4 Measurement of proliferation inhibition of cells

HUVECs were seeded onto gelatin-coated 24-multiwell plates at a density of 1.0×10^4 cells/cm^2 in HUVEC growth media. After 24 h incubation at 37°C in a 5% CO_2 incubator, each component of propolis was added to the wells under sterile conditions and the cells were further cultured for 3 days. The number of cells was counted with a Coulter Counter (Coulter Electronics, Hialeah, USA).

2.5 Determination of antioxidant activity

2.5.1 Free radical-scavenging activity on DPPH

The reaction mixture contained 2 mL of ethanol, 125 μM DPPH, and test samples. After 1 h incubation at room temperature, the absorbance was recorded at 517 nm. Control solution contained only ethanol and DPPH. Results were expressed as a percentage decrease relative to control values [36, 37]. Identified constituents were evaluated at a final concentration of 20 μg/mL, and VE and BHT at the same concentration were used as the reference samples.

2.5.2 FRAP assay

The FRAP reagent contained 2.5 mL of a 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma) solution in 40 mmol

HCl plus 2.5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6 [38]. Aliquots of 100 μL of each component of propolis were mixed with 3 mL FRAP reagent and the absorbance of the reaction mixture at 593 nm was measured spectrophotometrically after incubation at room temperature for 3 min. The FRAP activity was calculated by the equation:

$$\mathrm{FA} = \left(\frac{A_\mathrm{C}}{A_\mathrm{A}} \right) \times 100$$

where FA is the FRAP activity, A_C is the absorbance of the each component, A_A is the absorbance of the ascorbic acid, 100 is the concentration of the ascorbic acid (100 μg/mL). VE and BHT at the same concentration were used as the reference samples. Results were expressed as micrograms per milliliter of ascorbic acid equivalent.

3 Results

3.1 Various components from propolis inhibit HUVEC tube formation, an *in vitro* model of angiogenesis

Figure 1 shows the chemical structures of the main components from propolis. During normal tube formation, the endothelial cells migrated and gathered together, then became elongated and adhered to each other to form a network of capillary-like tubes (Fig. 2). We examined the inhibitory effect of ten components from propolis on such tube formation (Fig. 3). Acacetin (a) treatment had very little inhibitory effect on tube formation and the endothelial cells were able to form capillary-like tubes normally. Apigenin (b) had a mild inhibitory effect. It slightly reduced the width of the tubes at 3.13 μg/mL, further reduced the width of the tubes and caused partial fragmentation of the network at 12.5 μg/mL and completely inhibited elongation of the cells at 50 μg/mL. Artepillin C (c) had a similarly mild inhibitory effect. Caffeic acid phenethyl ester (d) had a strong inhibitory effect on tube formation. It disturbed tube morphology and caused slight fragmentation of the network at 3.13 μg/mL. It almost completely inhibited elongation of the cells at 12.5 μg/mL. It induced cell death of most cells at 50 μg/mL. Chrysin (e) had a weak inhibitory effect and the endothelial cells were able to form capillary-like tubes normally at 3.13 and 12.5 μg/mL. It slightly reduced the width of the tubes and caused partial fragmentation of the network at 50 μg/mL. Similarly, *p*-coumaric acid (f) had very little inhibitory effect with a slight difference. It reduced the width of the tubes slightly without causing fragmentation of the network between 3.13 and 50 μg/mL. Galangin (g), kaempferol (h), and pinocembrin (i) had a mild inhibitory effect similar to that of apigenin and artepillin C. Quercetin (j) had the strongest inhibitory effect among propolis ingredients we tested. It reduced the width of the tubes and caused extensive fragmentation of the network at

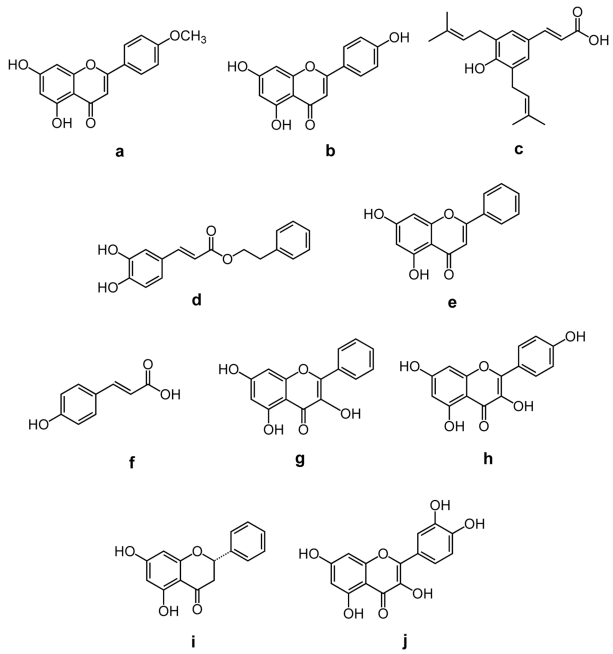

Figure 1. Structures of the main components from propolis: (a) acacetin; (b) apigenin; (c) artepillin C; (d) caffeic acid phenethyl ester; (e) chrysin; (f) *p*-coumaric acid; (g) galangin; (h) kaempferol; (i) pinocembrin; (j) quercetin.

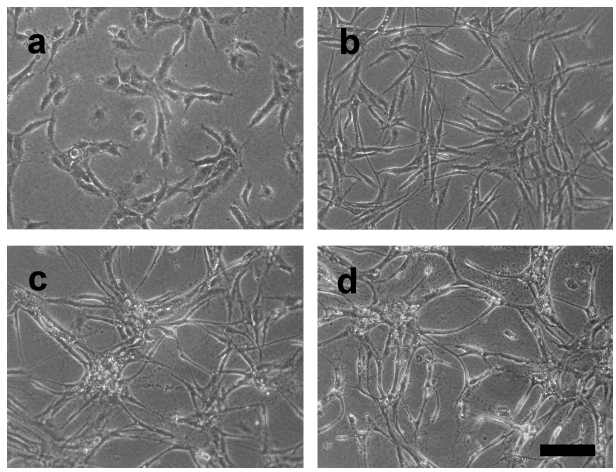

Figure 2. Tube formation of endothelial cells. Cells were sandwiched between two layers of collagen gel at a density of 6.0×10^4 cells/cm^2 and induced to form blood vessel-like tubes. The endothelial cells migrated and started to gather together 12 h after the induction of tube formation. Twenty-four hours after induction, the endothelial cells became elongated and adhered to each other to form a web-like structure. Thirty-six to forty-eight hours after induction, the cells formed a network of capillary-like tubes and each tube was composed of multiple cells with visible lumen inside. The cells adhered to each other very tightly and the boundary between them became unclear. (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h. The experiment was repeated three times and representative data are shown. Scale bar, 100 μm.

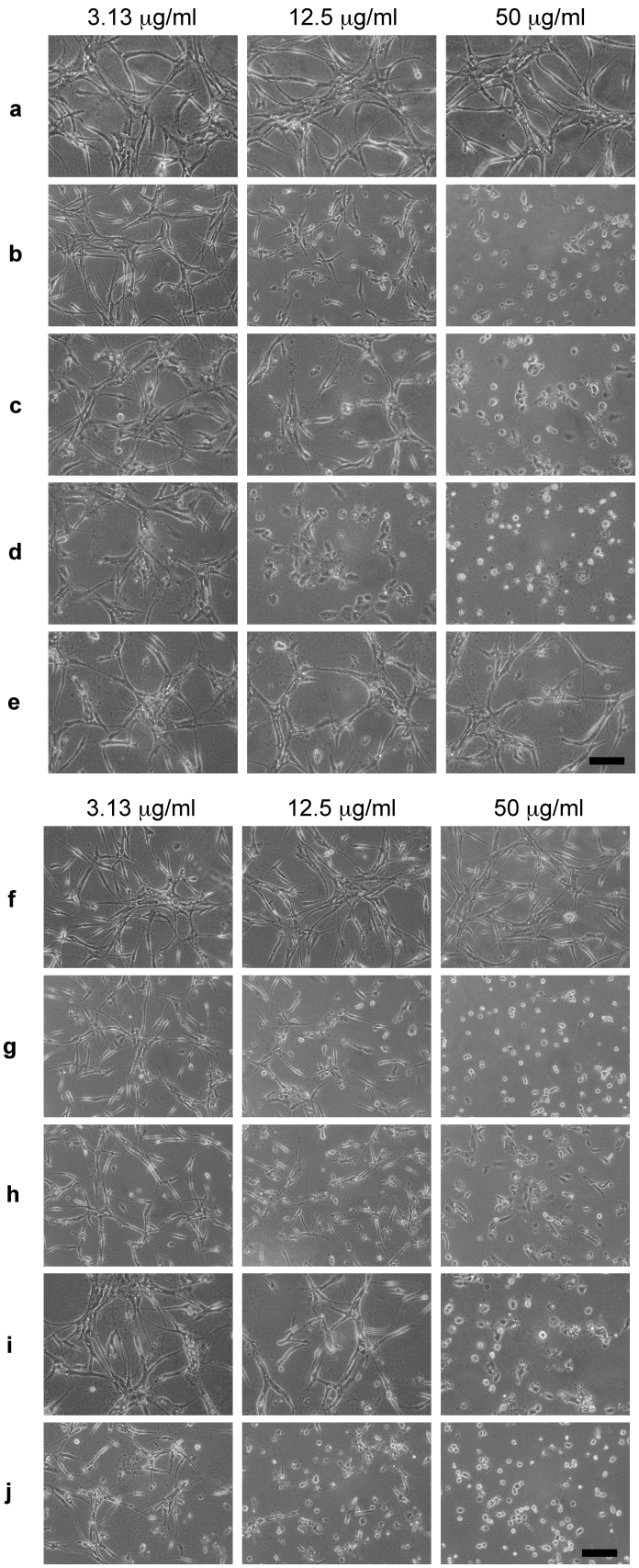

Figure 3. Inhibitory effects of various components from propolis on tube formation of endothelial cells. HUVECs were seeded and cultured between two collagen gel layers as described earlier in the presence of various concentrations of each component: (a) acacetin; (b) apigenin; (c) artepillin C; (d) caffeic acid phenethyl ester; (e) chrysin; (f) *p*-coumaric acid; (g) galangin; (h) kaempferol; (i) pinocembrin; (j) quercetin. The experiment was repeated three times and representative data are shown. Scale bar, 100 μm.

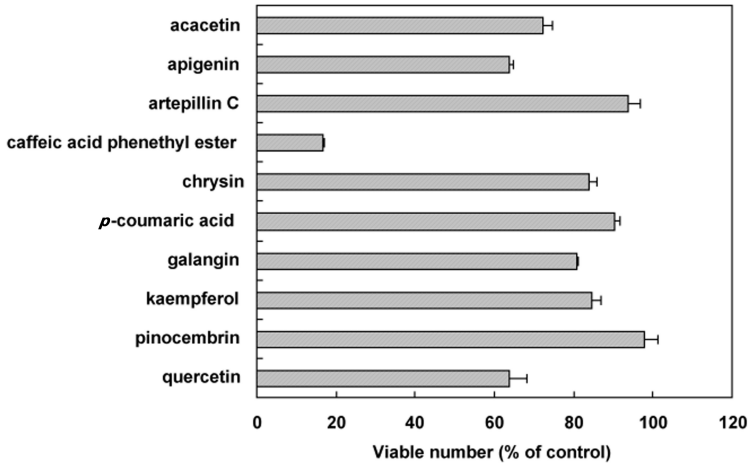

Figure 4. Inhibitory effects of various components from propolis on growth of endothelial cells. Cells were seeded on gelatin-coated plates at a density of 1.0×10^4 cells/cm^2 in 1.0 mL of HUVEC growth media. After 24 h incubation at 37°C in a 5% CO_2 incubator, each component was added to the wells at the indicated concentrations and the cells were cultured further for 3 days before conducting cell counts. Values are expressed as means ± SE ($n = 3$).

3.13 μg/mL. It almost completely inhibited elongation of the cells and induced cell death to nearly half of them at 12.5 μg/mL. It further induced cell death to most cells at 50 μg/mL. Thus, the ten compounds showed varying degrees of inhibitory effects in a concentration-dependent manner, except for acacetin and *p*-coumaric acid. They were classified into five classes based on their inhibitory activities on tube formation. Quercetin (j) was the strongest of all and caffeic acid phenethyl ester (d) was the second strongest. Apigenin (b), artepillin C (c), galaingin (g), kaempferol (h), and pinocembrin (i) were in the third strongest group with apparent inhibitory activities. On the other hand, chrysin (e) had a very weak inhibitory activity while acacetin (a) and *p*-coumaric acid (f) had very little to no inhibitory activities.

3.2 Inhibition of HUVEC proliferation, another *in vitro* model of angiogenesis, by various components from propolis

We then examined the effects of various components from propolis on endothelial cell proliferation, another *in vitro* model of angiogenesis (Fig. 4). All ten components from propolis significantly suppressed the proliferation of HUVECs in a concentration-dependent manner (3.13–50 μg/mL, data of only 3.13 μg/mL are shown).

At the lowest concentration of 3.13 μg/mL, caffeic acid phenethyl ester (d) showed an exceptionally strong inhibition with the inhibition rates of about 80% while all the other components had inhibition rates of less than 40%. Apigenin (b) and quercetin (j) showed the second strongest inhibition with inhibition rates of about 36%. The remaining components had inhibition rates of about 28% (acacetin (a)), about 15–20% (galangin (g), chrysin (e), and kaempferol (h)) and less than 10% (*p*-coumaric acid (f), artepillin C (c), and pinocembrin (i)). It should be noted that artepillin C (c) and pinocembrin (i) had very little inhibitory effects on HUVEC growth while acacetin (a) showed the fourth strongest inhibition rate.

3.3 DPPH free radical-scavenging activity of various components from propolis

Because the free radical-scavenging activity of antioxidants is considered to be due to their hydrogen-donating ability, we used a method based on the reduction of DPPH, a stable-free radical, to evaluate the antioxidant activity of various components of propolis [37, 39]. DPPH has been widely used to test the free radical-scavenging activity of various samples [40, 41]. We evaluated the free radical-scavenging activity of various components from propolis and the reference samples (BHT and VE) at a final concentration of 20 μg/mL (Fig. 5).

Various components from propolis showed free radical-scavenging activity to varying degrees. As shown in Fig. 5, caffeic acid phenethyl ester (d), kaempferol (h), and quercetin (j) had strong DPPH free radical-scavenging activity, over 90%. Artepillin C (c) had the next highest DPPH free radical-scavenging activity of 86.1%. Galangin (g) was the only member of the third highest group with the DPPH free radical-scavenging activity of 66.9%. The remaining components showed very weak DPPH free radical-scavenging activities, *p*-coumaric acid (f) being the only member of the fourth group at 23.7%. Acacetin (a), apigenin (b), chrysin (e), and pinocembrin (i) formed a group with the least DPPH free radical-scavenging activity at about 15%.

3.4 FRAP activity of various components from propolis

Measurement of reducing ability of the antioxidant property was performed using the FRAP method. The procedure described by Benzie and Strain [38] was followed. The principle of this method is based on the reduction of a ferric–tripyridyl–triazine complex to its ferrous, colored form in the presence of antioxidants.

Most of the various components from propolis showed FRAP activity (Fig. 6). Caffeic acid phenethyl ester (d) and quercetin (j) had strong FRAP activities of over 200 μg/mL.

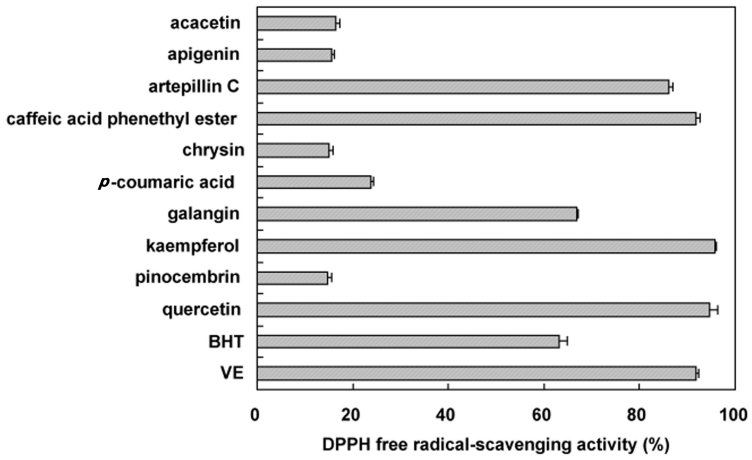

Figure 5. DPPH free radical-scavenging activity of various components from propolis. Means and SDs are indicated ($n = 3$).

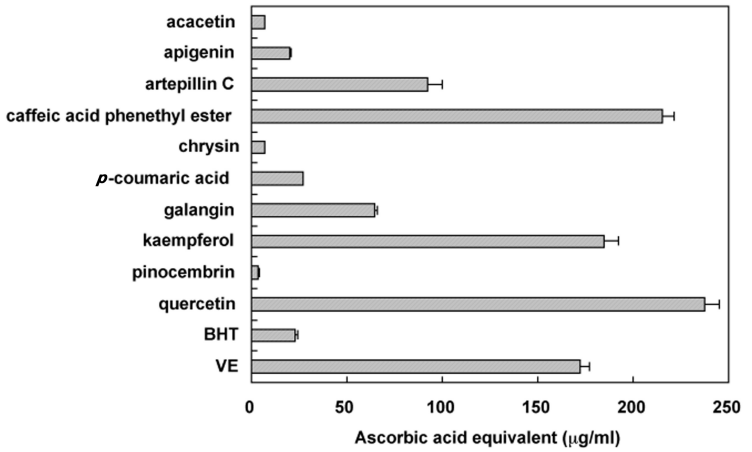

Figure 6. FRAP activity of various components from propolis. Means and SDs are indicated ($n = 3$).

Kaempferol (h) had the next strongest FRAP activity of 184.4 μg/mL. The remaining components showed relatively weak FRAP activities. Artepillin C (c) and galangin (g) had mild FRAP activities of 92.0 and 64.3 μg/mL, respectively. The next group consisted of apigenin (b) and *p*-coumaric acid (f) with FRAP activity of about 20–25 μg/mL. However, acacetin (a), chrysin (e), and pinocembrin (i) exhibited weak FRAP activities of 6.7, 7.0, and 3.3 μg/mL, respectively. Three of the components from propolis with high DPPH free radical-scavenging activity, caffeic acid phenethyl ester (d), kaempferol (h), and quercetin (j) showed very strong FRAP activity. While most of the components with mild to weak DPPH free radical-scavenging activity had comparable FRAP activity, acacetin (a), chrysin (e), and pinocembrin (i) had exceptionally low FRAP activity.

4 Discussion

In this study, we analyzed the antiangiogenic and antioxidant activities *in vitro* of various components from propolis. Although some investigators have reported that propolis can suppress tumor growth both *in vitro* and *in vivo* [14, 16, 20], the actual mechanisms of these effects are not yet fully understood. As previously reported, one possible explanation for such tumor suppressing effects of propolis is its antiangiogenic activity [25, 42]. In order to further elucidate the antiangiogenic mechanisms of propolis components in detail, we investigated the correlation between antiangiogenic activity and antioxidant activity of ten propolis components.

When the areas of all tubes constructed in the 2-D culture model were observed, several components from propolis were shown to possess strong inhibitory effects on tube formation of HUVECs in a concentration-dependent manner: quercetin (j) > caffeic acid phenethyl ester (d) > apigenin (b) = artepillin C (c) = galangin (g) = kaempferol (h) = pinocembrin (i). On the other hand, acacetin (a), chrysin (e), and *p*-coumaric acid (f) showed very weak to no detectable inhibitory effects on the capillary-like tube formation. These components from propolis were also shown to possess strong inhibitory effects on cell proliferation: caffeic acid phenethyl ester (d) > apigenin (b) ≥ quercetin (j) > acacetin (a) > galangin (g) > chrysin (e) ≥ kaempferol (h). On

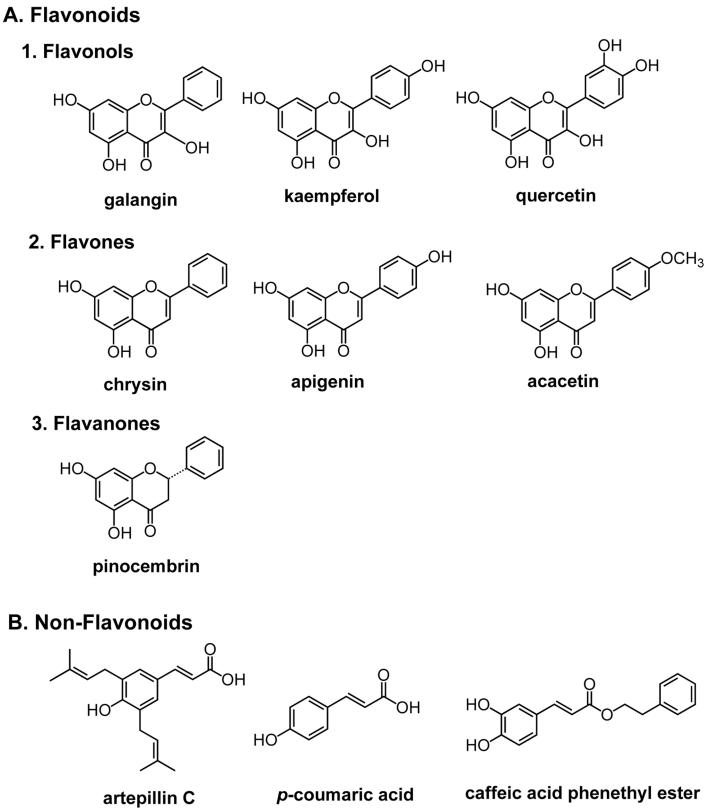

Figure 7. Classification of the main components from propolis based on differences in molecular backbone structure.

the other hand, *p*-coumaric acid (f), artepillin C (c), and pinocembrin (i) had very little inhibitory effects on cell proliferation.

Polyphenolic compounds are known to inhibit the growth of normal and cancer cells. For instance, it has recently been reported that artepillin C (c), caffeic acid phenethyl ester (d), and quercetin (j) have antiproliferative activity of human fibroblasts, endothelial, and tumor cells in a concentration-dependent manner [16, 43]. Quercetin (j) is an important constituent of the flavonoid family and is found in many fruits and vegetables. Various pharmacological activities of quercetin (j) have been demonstrated including antioxidant effects by scavenging free radicals, prevention of atherosclerosis, chronic inflammation, and anticancer activities. Caffeic acid phenethyl ester (d) has also been reported to possess preferential cytotoxicity on tumor cells. We would like to investigate whether these compounds exert such growth inhibitory effects on two different cell types, tumor cells and endothelial cells, through a similar mechanism at the molecular level.

We then used two assay systems for evaluating the *in vitro* antioxidant activity of various components from propolis. The antiangiogenic activity shown in Fig. 3 seemed to correlate well with that shown in Figs. 5 and 6. Components from propolis with high antiangiogenic activity, such as artepillin C (c), caffeic acid phenethyl ester (d), galangin (g), kaempferol (h), and quercetin (j), also had high antioxidant activity. The most convincing explanation for the correlation between antioxidative activity and antiangiogenic activity can be found in the role of superoxide and hydrogen peroxide in angiogenic signal transduction. In response to angiogenic factors such as VEGF and PMA, NADPH oxidase has been reported to produce superoxide and, subsequently, its metabolite hydrogen peroxide, which then activate multiple intracellular signaling pathways that lead to proliferation, migration, and tube formation of endothelial cells [44, 45]. However, when examined in detail, our results clearly show that strong antioxidant activity does not necessarily translate into strong antianigiogenic activity, although that seems to be the case most of the time. For instance, although caffeic acid phenethyl ester (d) and quercetin (j) possessed the strongest antiangiogenic activity and the strongest antioxidant activity at the same time, kaempferol (h), while showing the third strongest antioxidant activity among the ten compounds tested, had only mild antiangiogenic activity which was comparable to those of apigenin (b) and pinocembrin (i), both of which possessed very weak antioxidative activities. In addition to a few exceptions to the correlation between antioxidant and antiangiogenic activities, it has been recently reported that reactive oxygen species (ROS) generated by redox-silent analogs of vitamin E which target mitochondria can inhibit

angiogenesis by inducing apoptosis to endothelial cells [46, 47]. Since ROS generated by GF stimulation and ROS generated by mitochondria destabilization could have completely opposite effects, the role of ROS in angiogenesis needs to be carefully evaluated.

When seven flavonoids were compared based on their structural variations, it became clear that flavonols tended to show very strong antiangiogenic activity that seemed to be correlated to their strong antioxidant activity while flavones possessed relatively high antiangiogenic activity with only limited degree of antioxidative activity (Fig. 7). Among the three remaining nonflavonoids, artepillin C (c) and caffeic acid phenethyl ester (d) possessed strong antiangiogenic activity that seemed to be correlated to their strong antioxidant activity while *p*-coumaric acid (f) possessed very little antiangiogenic activity albeit its not-so-weak antioxidative activity. Based on these observations, it can be concluded that antioxidant activity and antiangiogenic activity of flavonoids strongly depend on their structure and features other than antioxidation. Flavonoids have been suggested to have several potential health benefits due to their antioxidant activities, which are attributed to the presence of phenolic hydroxyl (–OH) moieties on the structure [48, 49]. In addition to –OH moieties in the structural arrangements of flavonols, the resonance of electrons between A- and B-rings is very important for their antioxidant and biological activities. Bors *et al*. [50] reported that the *o*-dihydroxy phenyl ring is an important structure for the antioxidant activity of flavonoids. Kim *et al*. [51] also reported that the numbers of –OH moieties in this group of flavonols – myricetin, quercetin (j), kaempferol (h), and galangin (g), all with different numbers of –OH moieties – are important for their toxicity, in their antioxidant activity and, to some extent, in their effect on modulating endothelial cell angiogenesis, expression of adhesion molecules, and cell–cell adhesion. Thus, these compounds appeared to be potent candidates for chemopreventive agents and their antiangiogenic activity may be associated with their antioxidant activity. On the other hand, we must further investigate how these compounds exert antiangiogenic effects on endothelial cells apart from their antioxidative effects.

In this study, we found a correlation between antiangiogenic activity and antioxidant activity in various components from propolis. The components from propolis such as artepillin C (c), caffeic acid phenethyl ester (d), galangin (g), kaempferol (h), and quercetin (j) not only had strong antiangiogenic activity but also had strong antioxidant activity. These components might be responsible, at least in part, for the antiangiogenic activity of propolis *in vivo*. We are studying further the mechanism of inhibition of angiogenesis by various components of propolis at cellular and molecular levels.

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