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Inhibitory Actions of Bilberry Anthocyanidins on Angiogenesis

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The aim of this study was to examine the antiangiogenic properties and antioxidant activities (a) of the main anthocyanidins (delphinidin, cyanidin and malvidin) found as constituents in *Vaccinium myrtillus* (bilberry) anthocyanosides (VMA) and (b) of *N*-acetyl-L-cysteine (NAC). Each of these anthocyanidins concentration-dependently inhibited vascular endothelial growth factor (VEGF)-induced tube formation in a co-culture of human umbilical vein endothelial cells (HUVECs) and fibroblasts, the effect of each anthocyanidin being significant at 3 and/or 10 μM, while NAC significantly inhibited such tube formation at 1 mM (the only concentration tested). Moreover, each anthocyanidin (0.3–10 μM) and NAC (1–1000 μM) concentration-dependently scavenged the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The inhibitory effects against angiogenesis were similar among the anthocyanidins, as were those against the DPPH radical. Moreover, their radical-scavenging effects were induced by concentrations that were at or below those that induced their antiangiogenic effects. These findings indicate that the inhibitory effect of VMA on angiogenesis may depend on those of its main constituent anthocyanidins (delphinidin, cyanidin and malvidin), presumably via antioxidant effects. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: antioxidant; cyanidin; delphinidin; malvidin.

INTRODUCTION

Angiogenesis is defined as the generation of new vascular networks from existing blood vessels, with physiological angiogenesis being observed in endometrial, ovarian and wound healing. However, abnormal angiogenesis has been reported to lead to retinal diseases [for example, age-related macular degeneration (AMD), diabetic retinopathy (DR) and neovascular glaucoma] and to aid the progress of invading cancerous cells (Aiello *et al*., 1994; Facker *et al*., 2006; Nguyen *et al*., 1996). Angiogenesis involves multiple steps: [1] detachment of pre-existing mural pericytes (vascular destabilization), [2] degeneration of the extracellular matrix, [3] migration of endothelial cells, [4] proliferation of endothelial cells, [5] tube formation and [6] reattachment of pericytes (vascular stabilization).

Vaccinium myrtillus (bilberry), a member of the Ericaceous family, grows in the mountains and forests of Europe and North America. *Vaccinium myrtillus* extracts containing 15 different anthocyanins (Baj *et al*., 1983; Nakajima *et al*., 2004) have been shown to possess potent antioxidant properties (Nakajima *et al*., 2004), to stabilize collagen fibers (Morrazzoni and Bombardelli, 1996), to promote collagen biosynthesis (Morrazzoni and Bombardelli, 1996) and to inhibit platelet aggregation (Morrazzoni and Magistretti, 1990). Animal studies have demonstrated VMA to be of benefit in improving vascular tone, blood flow and for vasoprotection (Lietti *et al*., 1976; Colantuoni *et al*., 1991). When administered to healthy subjects or to patients with visual disorders, VMA induce significant improvements in night vision (Morrazzoni and Bombardelli, 1996). Hence, VMA have been utilized as a popular edible aid or supplement for asthenopia.

Recently, it was reported that VMA reduced both *in vitro* and *in vivo* angiogenesis (Matsunaga *et al*., 2007). Further, it was indicated that its effect may be due in part to reductions in cell proliferation and migration through inhibitions of both phosphorylated extracellular signal-regulated kinase 1/2 (ERK 1/2) and serine/threonine protein kinase family protein kinase B (Akt) (Matsunaga *et al*., 2007). In addition, it was demonstrated that the main anthocyanidin constituents (delphinidin, cyanidin and malvidin) of VMA have inhibitory effects on lipid peroxidation and also neuroprotective effects (Matsunaga *et al*., 2008). However, it has not been established which constituents are the responsible substance(s) for the antiangiogenic effects of VMA.

Previous studies have demonstrated that oxidative stress is associated with increased production of VEGF under *in vitro* conditions, and there is thought to be an upregulation of VEGF expression during diabetes (Ellis *et al*., 1998; Obrosova *et al*., 2004; Chade *et al*., 2004).

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Furthermore, an antioxidant substance, NAC, inhibits the VEGF-induced migration of HUVECs, and its effect is mediated by an inhibition of the Src (cytoplasmic protein tyrosine kinase) signal pathway (Lin *et al*., 2003). Previous studies have established that VMA have powerful antioxidant effects, and at least some constituents of VMA might be expected to inhibit angiogenesis via such effects (Nakajima *et al*., 2004; Matsunaga *et al*., 2007, 2008). In previous studies it was indicated that bilberry inhibits H_2O_2-induced expression of VEGF from the keratinocyte via antioxidant effects (Roy *et al*., 2002; Bagchi *et al*., 2004). In order to investigate a relation between these (antiangiogenic and antioxidant) effects, the *in vitro* effects of the three major constituents of VMA (delphinidin, cyanidin and malvidin) were examined, and also the effects of NAC, against both VEGF-induced angiogenesis and the DPPH radical.

MATERIALS AND METHODS

Materials. Delphinidin, cyanidin and malvidin (Fig. 1) were purchased from Extrasynthese (Genay Cedex, France). VMA was kindly gifted by Fushimi Chemical Co. Ltd (Kyoto, Japan). DPPH and the oxygen-scavenger NAC were purchased from Wako (Osaka, Japan), while VEGF was from Kurabo (Osaka, Japan).

Tube formation assay. An angiogenesis assay kit (Kurabo) was used according to the manufacturer's instructions. Briefly, HUVECs co-cultured with fibroblasts were cultivated in the presence or absence of various concentrations of test drugs (or their vehicle) plus VEGF (10 ng/mL). After 11 days, the cells were fixed in 70% ethanol. The cells were incubated with diluted primary antibody (mouse anti-human CD31; 1:4000) (Kurabo) for 1 h at 37 ºC, and with the secondary antibody (goat anti-mouse IgG alkaline phosphatase-conjugated antibody; 1:500) (Kurabo) for 1 h at 37 ºC, and visualization was achieved using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Kurabo). Images were obtained from five different fields (5.5 mm^2 per field) for each well, and the tube area, length, joints and paths were quantified using an angiogenesis image analyser Ver.2 (Kurabo). The tube area is the total area measured two-dimensionally. The tube length is the total length of the various tubes. A joint is where two different tubes intersect (i.e. a 'branch point'). Paths are the total number of pieces of tube branching from all joints.

Measurement of DPPH radical-scavenging activity. Radical-scavenging activity was measured by means of a DPPH assay. DPPH is a stable free radical with a purple color (absorbed at 517 nm). If free radicals are scavenged, DPPH changes color to colorless. Therefore, the DPPH assay is used to show free radical scavenging activity. VMA, delphinidin, cyanidin, malvidin and NAC were dissolved and diluted in ethanol at various concentrations, then 0.025 mg/mL DPPH in ethanol was added, and the whole was left to stand for 30 min at room temperature. This was followed by measurement of the absorbance of the resulting solution at 517 nm using a spectrophotometer (Shimadzu, Kyoto, Japan).

Statistical analysis. Data are presented as the mean ± SEM. Statistical comparisons were made using a one-way ANOVA followed by Dunnett's multiple-comparison test, or a Student's *t*-test. A value of $p < 0.05$ was considered to indicate statistical significance.

RESULTS

Anthocyanidins and NAC inhibited VEGF-induced tube formation in HUVEC co-cultured with fibroblasts

Representative images of the tube formation induced by the addition of VEGF plus either an anthocyanidin (delphinidin, cyanidin or malvidin) or NAC are shown in Fig. 2. Each of the three anthocyanidins (1–10 μM) concentration-dependently inhibited VEGF-induced tube formation, and quantitative analysis showed that each of them (at 3 μM and/or at 10 μM) and NAC (at 1 mM) significantly inhibited tube formation (tube area, length, joints and paths) (Fig. 3). These effects were quite similar among the three anthocyanidins at 1–10 μM.

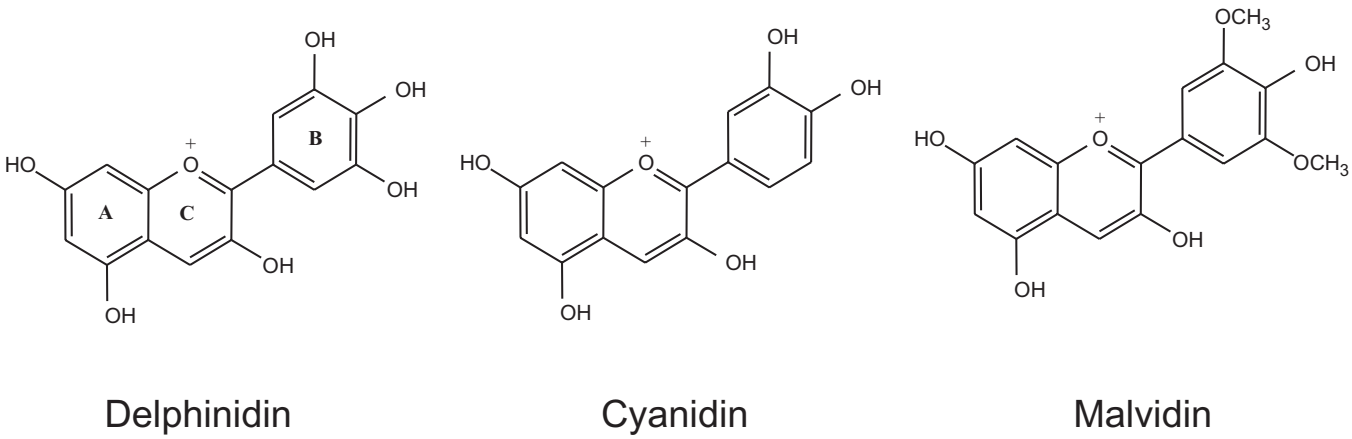

Figure 1. Chemical structures of delphinidin, cyanidin and malvidin. Anthocyanidins, a subclass of flavonoids, are defined by their three-ring ($C_6C_3C_6$) structure consisting of two benzene rings (**A** and **B** rings) flanking an oxygen-containing pyran ring (**C** ring).

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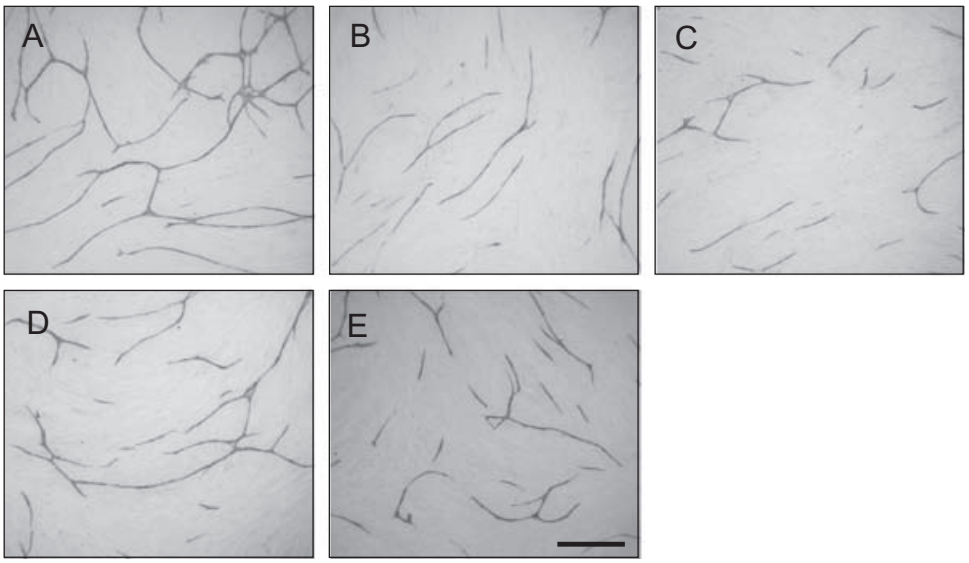

Figure 2. Effects of delphinidin, cyanidin, malvidin and NAC on vascular endothelial growth factor (VEGF)-induced tube formation in HUVECs. Representative photographs of tube formation (A) vehicle, (B) delphinidin at 10 μM, (C) cyanidin at 10 μM, (D) malvidin at 10 μM, (E) NAC at 1 mM. Scale bar = 500 μm. HUVECs were co-cultured with human fibroblasts (as described in Materials and Methods), and incubated for 11 days with one of the above in the presence of VEGF (10 ng/mL).

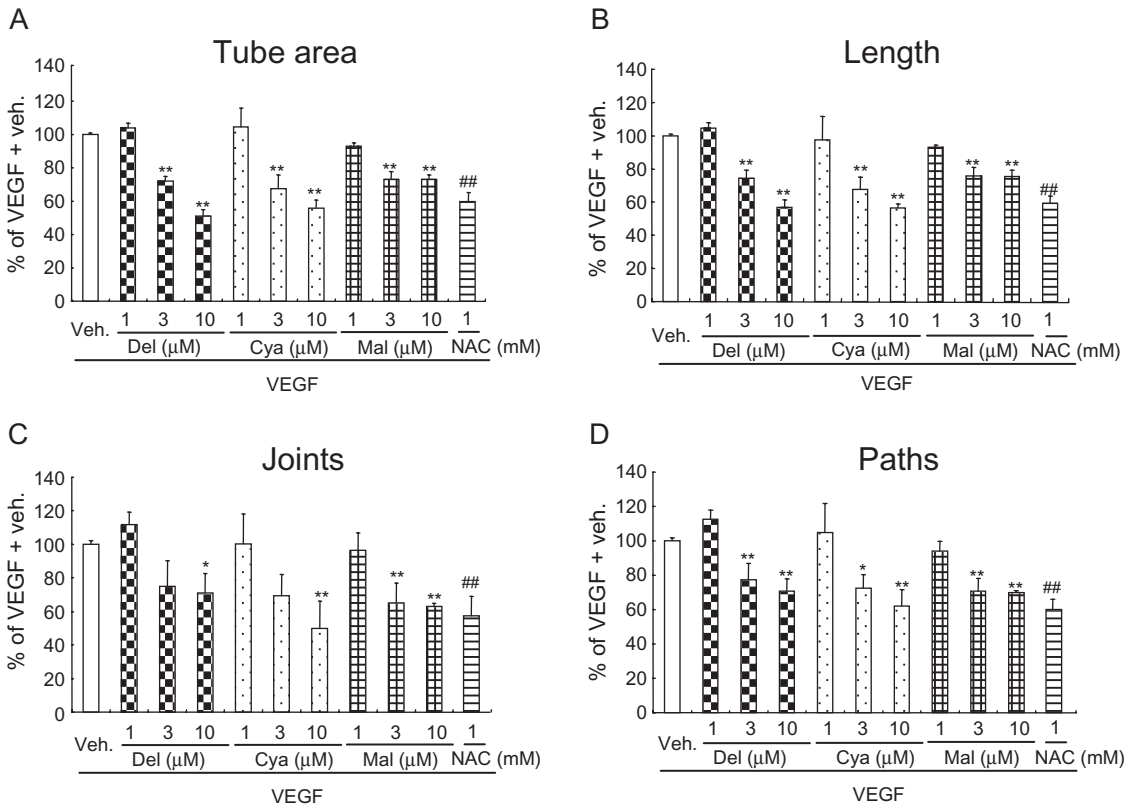

Figure 3. Effects of anthocyanidins and NAC on VEGF-induced tube formation. Tube formation was observed in five randomly chosen fields, and tube area (A), length (B), joints (C) and paths (D) were evaluated using an angiogenesis image analyser. Data are shown as mean ± SEM. (VEGF + vehicle: $n = 9$, VEGF + the indicated compound: $n = 3$). Veh., vehicle; Del, delphinidin; Cya, cyanidin; Mal, malvidin. * $p < 0.05$, ** $p < 0.01$ vs vehicle (Dunnett's multiple-comparison test). ## $p < 0.01$ vs vehicle (Student's *t*-test).

In addition, the inhibitory effects of the three anthocyanidins at 10 μM were approximately the same as those of NAC at 1 mM. However, the inhibitory effect of malvidin at 10 μM on the VEGF-induced tube area and length was weaker than those of delphinidin, cyanidin at 10 μM and NAC at 1 mM.

VMA, anthocyanidins and NAC exhibited radical-scavenging activity against DPPH

The radical-scavenging activities of VMA, the three anthocyanidins and NAC were measured using a DPPH assay. VMA, delphinidin, cyanidin, malvidin and NAC

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each concentration-dependently exhibited radical-scavenging effects against the DPPH radical, their 50% inhibitory concentration (IC_{50}) values being 2.7 (2.1–3.4, 95% confidence limits) μg/mL, 8.5 (7.4–10.0) μM, 5.7 (4.9–6.6) μM, 8.4 (6.7–10.9) μM and 36.2 (26.5–49.6) μM, respectively. All three anthocyanidins at 1–10 μM significantly scavenged the DPPH radical, with the effects of cyanidin and malvidin also being significant at 0.3 μM (Fig. 4). These effects were similar among the three anthocyanidins (Fig. 4), while the radical-scavenging effect of NAC at 1 mM was 1.6–2.3 times stronger than those exerted by the anthocyanidins at 10 μM (Fig. 4).

DISCUSSION

In the present study, it was found that the three main anthocyanidin constituents of VMA (delphinidin, cyanidin and malvidin) were about equal in their inhibitory effects on VEGF-induced angiogenesis and also in their effects against the DPPH radical. Moreover, their effects against the DPPH radical were induced by concentrations that were the same as, or lower than, those that induced their antiangiogenic effects. This suggests that their inhibitory effects against angiogenesis may be mediated at least in part by reductions in oxidative stress.

Anthocyanidins may be broken down within a few hours in the culture medium since they are unstable to heat, light and pH. Anthocyanidins in the culture medium were exchanged on days 1, 4, 7 and 9 during 11 days of the tube formation assay. After the medium exchange, anthocyanidins were capable of inhibiting VEGF-induced intracellular signaling within a few hours (before degradation of anthocyanidin). It was reported that VMA inhibits VEGF-induced activation of intracellular signaling, ERK1/2 (within 5 min) and Akt (within 10 min) (Matsunaga *et al*., 2007). It was considered that VMA can promptly inhibit the VEGF-induced signaling. On the other hand, Tsuda *et al.* reported that protocatechuic acid, metabolite of cyanidin 3-glucoside, scavenged free radicals (Tsuda *et al*., 1996). Probably, the radical scavenging effects of protocatechuic acid, a degradation product of cyanidin, inhibited the VEGF-induced tube formation.

VMA contain more than 20% of anthocyanidin (calculated as delphinidin), five anthocyanidins (cyanidin, delphinidin, petunidin, paeonidin and malvidin) being particularly prevalent, and these can be linked to a variety of saccharides, which may be glucose, galactose or arabinose. VMA contains 15 kinds of anthocyanin: delphinidin 3-*O*-arabinopyranoside, delphinidin 3-*O*-galactopyranoside, delphinidin 3-*O*-glucopyranoside, cyanidin 3-*O*-arabinopyranoside, cyanidin 3-*O*-galactopyranoside, cyanidin 3-*O*-glucopyranoside, petunidin 3-*O*-alabinopyranoside, petunidin 3-*O*-galactopyranoside, petunidin 3-*O*-glucopyranoside, malvidin 3-*O*-arabinopyranoside, malvidin 3-*O*-glucopyranoside, malvidin 3-*O*-galactopyranoside, paeonidin 3-*O*-arabinopyranoside, paeonidin 3-*O*-galactopyranoside and paeonidin 3-*O*-glucopyranoside. The constituents of VMA in this study were the same as in previous reports (Matsunaga *et al*., 2007, 2008), and 15 kinds of anthocyanins in VMA were confirmed by HPLC analysis (Matsunaga *et al*., 2008). The delphinidin and cyanidin contents of VMA were higher than those of the other anthocyanidins (Baj *et al*., 1983). Further, anthocyanin preparations containing delphinidin exhibit higher rates of absorption from the gut than the other anthocyanins in VMA (Talavéra *et al*., 2003). The availability of the hydroxyl group on the B ring (benzene ring on the right side of the structure in Fig. 1) of a given anthocyanidin (for example, delphinidin and cyanidin)

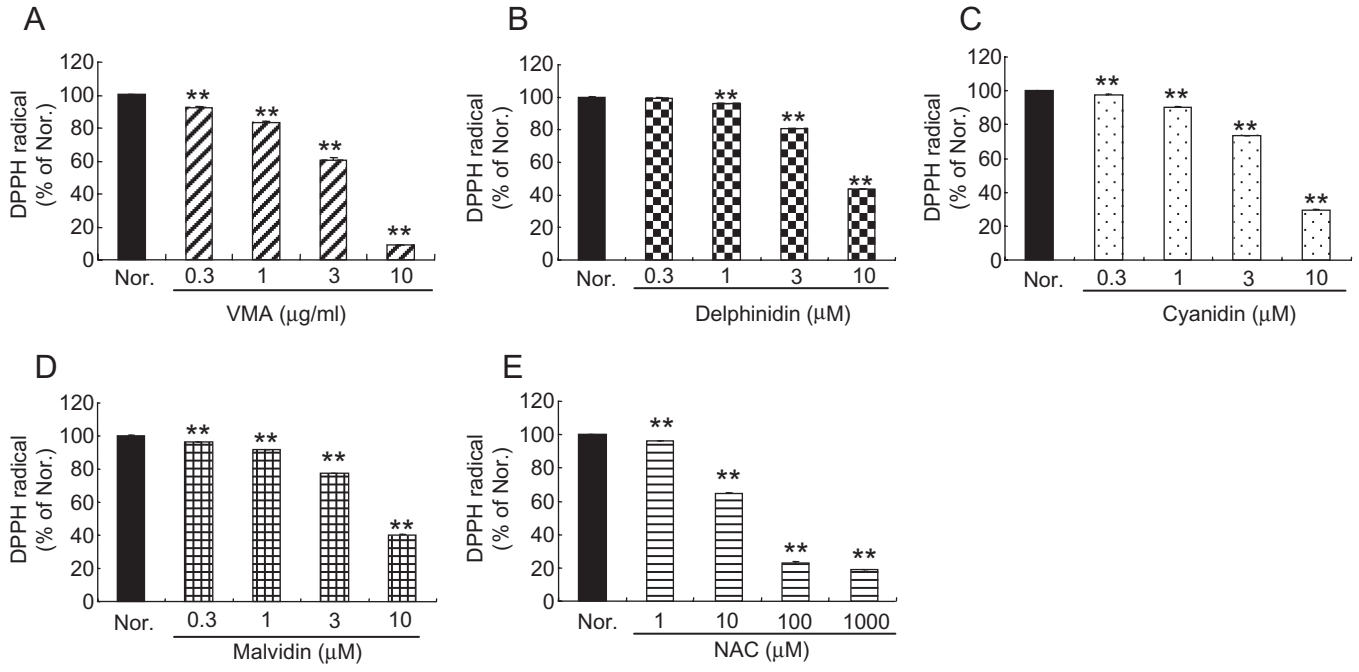

Figure 4. Radical-scavenging activities of VMA, delphinidin, cyanidin, malvidin and NAC. Following incubation of one of the above with DPPH for 30 min, the absorbance at 517 nm due to DPPH radical was determined. Data are shown as mean ± SEM. (n = 3 or 4). Nor., normal. ** $p < 0.01$ vs normal (Dunnett's multiple-comparison test).

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is fundamental to the antioxidant activity of these compounds because these hydroxyl moieties can donate hydrogen for the scavenging of radicals (Vinson *et al*., 1995; Torel *et al*., 1986). Indeed, the donated hydrogen serves to scavenge oxygen radicals such as superoxide and hydroxyl radicals (Torel *et al*., 1986). Previous studies have reported that malvidin-glucopyranoside inhibits the free radical-initiated peroxidation of linoleic acid (Rossetto *et al*., 2002; Tamura and Yamagami, 1994). Furthermore, it was reported that VMA and its main constituents (delphinidin, cyanidin and malvidin) inhibit both lipid peroxidation in the mouse forebrain and oxidative stress in neuronal cells (Matsunaga *et al*., 2008). As reported previously, it was confirmed that VMA and its main constituents can scavenge the DPPH radical (Kahkonen and Heinonen, 2003, and Fig. 4) in the present study. Taken together, the above findings indicate that VMA and its main constituents (cyanidin, delphinidin and malvidin) have antioxidant effects.

It was confirmed previously, by HPLC analysis, that VMA contains mainly delphinidin, cyanidin and malvidin (Matsunaga *et al*., 2008). At the concentration used in the present study (namely 10 μg/mL), VMA would contain delphinidin at 4.5 μM (15.1%), cyanidin at 2.6 μM (8.36%) and malvidin at 1.4 μM (5.43%) (calculated from Baj *et al*., 1983). For this reason, these anthocyanidins were used at 1–10 μM in the present *in vitro* studies. At 3 and/or 10 μM, they significantly inhibited VEGF-induced tube formation (Fig. 3). In a previous study, it was found that VMA at 30 μg/mL inhibited all four measured parameters of VEGF-induced tube formation (tube area, length, joints and paths being reduced to 30%, 33%, 27% and 33%, respectively, of control) (Matsunaga *et al*., 2007). In the present study, the inhibitory effects induced by anthocyanidins, even at 10 μM, were weaker than those of VMA at 30 μg/mL (tube area, length, joints and paths being reduced by the anthocyanidins to 51–73%, 56–75%, 49–70% and 62–71%, respectively, of control; Fig. 3). Therefore, the inhibitory effect of VMA on VEGF-induced angiogenesis may involve a summation, or a degree of interaction, among the effects of the constituent anthocyanins.

Reportedly, H_2O_2 stimulates angiogenesis *in vitro* (Yasuda *et al*., 2000), and the antiangiogenic effects of many compounds [such as ascorbic acid (Ashino *et al*., 2003), resveratrol (Lin *et al*., 2003) and NAC (Lin *et al*., 2003)] are achieved via their antioxidant properties. NAD(P)H oxidase, a major source of endothelial superoxide generation, is required for VEGF-induced endothelial cell proliferation and migration (Abid *et al*., 2000). Further, reactive oxygen species (ROS) derived from NAD(P)H oxidase mediate VEGF-induced endothelial cell proliferation and migration, and also VEGF receptor-2 (VEGFR-2) tyrosine phosphorylation (Ushio-Fukai *et al*., 2002). El-Remessy *et al.* suggested that peroxynitrite, a kind of ROS, is induced by VEGF, and VEGFR-2 is activated by the peroxynitrite. Further, they indicated that the addition of NAC at 1 mM inhibited approximately 40% VEGF-induced proliferation and tube formation of HUVEC (El-Remessy *et al*., 2007). Their data were similar to our data (Fig. 3). In the present study, VMA, delphinidin, cyanidin, malvidin and NAC all displayed both antiangiogenic and DPPH radical-scavenging effects (Figs 2–4; also see Matsunaga *et al*., 2007). Taken together, the above observations suggest that the inhibitory actions of VMA against angiogenesis may be due in part to the radical-scavenging effects of the constituent anthocyanins (including delphinidin, cyanidin and malvidin). However, the radical-scavenging effect of each anthocyanidin at 10 μM was weaker than NAC at 1 mM, although the inhibitory effect of VEGF-induced angiogenesis on each anthocyanidin at 10 μM and NAC at 1 mM was the same. Therefore, it is considered that the inhibitory effect of each anthocyanidin on angiogenesis cannot be attributed to the radical-scavenging effects alone. It has been reported that flavonoids (for example, flavane, flavanone and PD98059) inhibit the phosphorylation of ERK1/2 (Reiners *et al*., 1998). Probably, the chemical structure of flavonoids in anthocyanidin may affect the signal pathways in the cell.

In conclusion, the findings indicate that the anthocyanidins (delphinidin, cyanidin and malvidin) included in VMA both inhibit VEGF-induced angiogenesis and scavenge the DPPH radical. On the basis of these findings, it is suggested that the inhibitory effect of VMA on angiogenesis is mainly due to the effects of their major constituents, delphinidin, cyanidin and malvidin, and that the underlying mechanism may involve radical-scavenging activities.

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