**Letter to the Editor**

**Decrease of reactive oxygen species and reciprocal increase of nitric oxide in human dermal endothelial cells by Bidens pilosa extract: A possible explanation of its beneficial effect on livedo vasculopathy**

*Bidens pilosa* Linn. var. *radiata* (*B. pilosa*) is a tropical weed widely present in tropical and subtropical regions, including Miyako Island, Okinawa, Japan. This plant is used in various folk medicines and as a popular ingredient in herbal tea for their blood-pressure-lowering, liver-protective, and hypoglycemic effect [1,2]. In the therapeutic guidelines for vasculitis and vascular disorders of the Japanese Dermatological Association, *B. pilosa* tea is recognized as an effective remedy for the treatment of livedo vasculopathy [3,4].

The production of nitric oxide (NO) and reactive oxygen species (ROS) from endothelial cells (ECs) has been recognized as a key determinant of vascular homeostasis, regulating several physiological properties of the blood vessels [5]. Substances that decrease ROS or increase NO, or both, may be suitable candidates for therapeutic intervention of livedo vasculopathy. Certain anti-oxidative phytochemicals as well as pharmaceutical agents exert anti-oxidative signals after binding to aryl hydrocarbon receptor (AhR), which up-regulate anti-oxidative signaling pathways, such as nuclear factor-erythroid 2-related factor-2 (Nrf2) and NAD(P)H:quinone oxidoreductase 1 (Nqo1) [7]. However, the impact of *B. pilosa* extract on the regulation of ROS and NO in ECs has not been investigated. In this study, we examined the production of ROS and NO from human dermal ECs in the presence and absence of *B. pilosa* extract.

The dried powder of *B. pilosa* extract [2] was provided by Musashino Research Institute for Immunity Co., Ltd. (Okinawa, Japan). Human dermal ECs (Lonza, Walkersville, MD) were grown in Endothelial basal medium (Lonza) supplemented with hydrocortisone, hEGF, FBS, VEGF, hFGF-β, R3-IGF-1, ascorbic acid, heparine, and gentamicin/amphotericin-B. Immunofluorescence study was performed as previously described [7], and analyzed using D-eclipse confocal laser scanning microscope (Nikon, Tokyo, Japan). Quantitative real-time PCR (qRT-PCR) was performed as previously described [7]. ROS production was detected using carboxy-2,7'-dichlorofluorescein diacetate (carboxy-H2DCFDA) (molecular probes) [6], and analyzed using ImageJ software (the National Institutes of Health, Bethesda, MD). NO production was detected using diaminofluorescein-2 diacetate (DAF-2DA; Sekisui Medical, Tokyo, Japan). Small interfering (si)RNA targeted to AhR (si-AhR, si1200) and Nrf2 (si-Nrf2, si9492), and siRNA consisting of a scrambled sequence (si-control) were purchased from Ambion (Austin, TX, USA). ECs were incubated with HiPerFect Transfection

![Fig. 1](image-url)  
**Fig. 1.** *B. pilosa* extract up-regulated an anti-oxidative pathway through activation of AhR signaling. (A) Confocal laser scanning microscopic analysis of AhR staining in ECs. In unstimulated ECs, AhR staining was observed in the cytoplasm. Nuclear translocation of AhR was evident with 1μM BaP and 1mg/ml *B. pilosa* extract. (B) ECs were treated with medium only and various concentrations (0.01–1mg/ml) of *B. pilosa* extract, and qRT-PCR assay was performed for CYP1A1, Nrf2, and Nqo1 transcription. (C) CYP1A1, Nrf2, and Nqo1 induction by *B. pilosa* extract was canceled in si-AhR ECs. (D) Nqo1, but not CYP1A1, induction by *B. pilosa* extract was canceled in si-Nrf2 ECs. Data are presented as means ± SD (n = 3) (B–D). *p* < 0.05, determined by unpaired Student’s t-test.

**Keywords:** Livedo vasculopathy; *Bidens pilosa*; Aryl hydrocarbon receptor; Reactive oxygen species

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kit (Qiagen, Courtaboeuf, France), containing 5 nM siRNAs for 48 h before treatment with B. pilosa extract.

To determine whether B. pilosa extract activates AhR signaling, we first examined AhR nuclear translocation. AhR was mainly localized in the cytoplasm under unstimulated conditions (Fig. 1A). Following BaP (1 μM) treatment, AhR staining was observed mainly in the nucleus, indicating that BaP induced AhR nuclear translocation in ECs. Like BaP, B. pilosa extract (1 mg/ml) also induced nuclear translocation of AhR (Fig. 1A). Upon AhR activation, B. pilosa extract up-regulated the mRNA expression of CYP1A1, Nrf2 and Nqo1 (Fig. 1B). In addition, induction of CYP1A1, Nrf2, and Nqo1 was abolished in ECs transfected with si-AhR (Fig. 2A and B).

Fig. 2. (A and B) ROS production from ECs was detected by carboxy-H2DCFDA. ECs were treated with medium control or B. pilosa extract (1 mg/ml) in the presence or absence of TNF-α (10 ng/ml). Data are presented as means of fluorescence intensity of ROS ± SD (n = 3). The intensity of the medium control was set as 100%. *p < 0.05, determined by unpaired Student’s t-test. (C) ROS production from ECs treated with si-control, si-AhR or si-Nrf2 in the presence or absence with B. pilosa extract or TNF-α. TNF-α-induced ROS production was inhibited by B. pilosa extract. Knockdown of either AhR or Nrf2 restored the ROS inhibition by B. pilosa extract. (D) NO production was detected by DAF-2DA from ECs. Marked NO production was observed in ECs treated with B. pilosa extract. (E) Fluorescence intensity of NO was compared in ECs treated with medium control and B. pilosa extract. The intensity of medium control was set as 100% (n = 3). (F) NO production in ECs treated with B. pilosa extract and/or TNF-α were not affected by si-AhR.
si-AhR, indicating that B. pilosa extract-induced CYP1A1, Nrf2, and Nqo1 expression is AhR-dependent (Fig. 1C). Furthermore, Nqo1 induction, but not CYP1A1, was inhibited in ECs transfected with si-Nrf2 (Fig. 1D), verifying that the anti-oxidative NQO-1 induction is dependent on AhR-Nrf2 activation.

As the Nqo1 induction potently suppressed the ROS production in keratinocytes perturbed by TNF-α [7], we next examined whether the B. pilosa extract was feasible to inhibit the ROS production in TNF-α-treated ECs. Striking ROS production was observed in the presence of TNF-α (Peprotech, Rocky Hill, NJ), while B. pilosa extract significantly inhibited the TNF-α-induced ROS production in ECs (Fig. 2A and B). Furthermore, knockdown of either AhR or Nrf2 canceled the inhibitory effect of B. pilosa extract on TNF-α-induced ROS production (Fig. 2C). In addition, TNF-α-injured ECs lost their adhesiveness and detached from the plate, which was partially restored by B. pilosa extract (data not shown).

We further examined the effect of B. pilosa extract on the NO production in ECs. B. pilosa extract, per se, markedly and significantly enhanced the NO production compared with the medium control (Fig. 2D, E). In sharp contrast to the ROS production, knockdown of AhR did not affect the NO production of ECs by the B. pilosa extract (Fig. 2F).

ROS and NO play fundamental roles in maintaining the homeostasis of the vascular system. ROS potently activates platelets, leading to coagulation and thrombosis, which is associated with hypertension and coronary heart disease [8]. In contrast, NO is also a highly versatile molecule that exerts vasorelaxing effects, and also possesses antiplatelet, antithrombotic, and anti-inflammatory properties within the vasculature [5]. Although the exact etiology of livedo vasculopathy is still unknown, the disease is now widely believed to have a procoagulant pathogenesis frequently associated with autoantibodies such as anti-phospholipid antibodies and lupus anticoagulant [9]. Variable effectiveness of a range of antithrombotic agents and antiplatelet/anticoagulants, such as warfarin and heparin, has been documented [9]. Masuzawa et al. treated 14 cases of livedo reticularis with summer ulceration using B. pilosa tea for five years, and its preventative effect was observed in an average of 87% cases per year [3]. As B. pilosa has been reported to have potent antioxidant activities [10], we speculated that the mixture of phytochemical ingredients in B. pilosa extract may affect the NO and ROS production of ECs. Our results demonstrated that B. pilosa extract activated AhR and induced the subsequent Nrf2 and downstream anti-oxidative enzyme Nqo1 transcription in cultured human dermal ECs. ROS production of TNF-α-treated ECs was potently inhibited by the extract. In contrast, treatment with B. pilosa extract markedly up-regulated the production of NO via an AhR-independent mechanism. As the B. pilosa extract contains a series of phytochemicals, the possibility remains that the components responsible for ROS inhibition differ from those that enhance NO production. Although further assessments are necessary to prove the exact biomechanisms, B. pilosa extract may be a useful adjunct to treat microangiopathy through increasing the NO/ROS ratio, which supports the beneficial effects of B. pilosa tea for livedo vasculopathy in a clinical setting.

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References


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