Prev. Nutr. Food Sci. 2020;25(2):173-183
https://doi.org/10.3746/pnf.2020.25.2.173
pISSN 2287-1098 · eISSN 2287-8602

Combined Extract of *Vitis vinifera* L. and *Centella asiatica* Synergistically Attenuates Oxidative Damage Induced by Hydrogen Peroxide in Human Umbilical Vein Endothelial Cells

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ABSTRACT: Endothelial cell injury caused by oxidative stress is a critical factor in the initial stage of vascular diseases. Thus, identification of more effective antioxidants is a promising strategy to protect against endothelial cell injury. Recently, synergistic effects between phytochemicals have received renewed attention for their role in the treatment of various diseases. *Vitis vinifera* L. and *Centella asiatica* are well-known medicinal plants with various biological effects. However, the combination of the two has not previously been studied. Here, we investigated the effects of *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC), a standardized herbal blend comprising *V. vinifera* L. leaf extract (VE) and *C. asiatica* extract (CE), for its antioxidant activity and for the protection of endothelial cells against hydrogen peroxide (H_2O_2)-mediated oxidative damage in human umbilical vein endothelial cells (HUVECs). VCEC showed higher antioxidant activity than VE or CE in oxygen radical antioxidant capacity assays. In HUVECs, VCEC significantly suppressed increases in the production of intracellular reactive oxygen species, decreased levels of nitric oxide and vascular endothelial-cadherin, and increased endothelial hyperpermeability triggered by H_2O_2. Treatment with VE or CE alone ameliorated HUVEC injury in a pattern similar to VCEC, although their effects were significantly weaker than VCEC. Overall, VCEC exhibited a substantial synergistic effect on protecting endothelial cells against oxidative damage through its antioxidant activity. Therefore, VCEC could be developed as a potential agent for reducing the risk of vascular diseases related to oxidative stress.

Keywords: *Centella asiatica*, endothelial cell, oxidative stress, *Vitis vinifera*

INTRODUCTION

The vascular endothelium consists of a layer of endothelial cells in the inner surface of blood vessels and is a crucial barrier between the blood and tissue environments. The vascular endothelium plays a pivotal role in vascular permeability, maintenance of vascular tone and pressure, platelet aggregation, and thrombosis (Minami et al., 2019). Endothelial cells can be easily damaged by various factors, such as inflammatory cytokines and reactive oxygen species (ROS), which cause endothelial dysfunction that, in turn, initiate early processes of vascular diseases, including atherosclerosis, hypertension, and peripheral venous disease (Herrera et al., 2010; Burtenshaw et al., 2019).

In particular, oxidative stress caused by ROS is a critical factor in the pathogenesis of vascular diseases. In the endothelium, ROS are produced by diverse metabolic enzymes, such as reduced nicotinamide adenine dinucleotide phosphate oxidase, lipoxygenase, and xanthine oxidase (Sugamura and Keaney, 2011; Banerjee et al., 2019). Overproduction of ROS in vascular lesions disrupts the balance between cell survival and cell death signaling pathways, peroxidizes membrane lipids, and triggers the production of several inflammatory mediators (Irani, 2000; Zhang, 2008). As a result, endothelial permeability is increased, and inflammatory cells, such as monocytes, migrate to the endangium by binding to cell adhesion molecules, which is important during the development of vascular diseases (Bot et al., 2015). Therefore, antioxidant agents that attenuate the generation of ROS may have therapeutic applications in reducing endothelial cell damage. For example, vitamin E supplementation has been shown to reduce free radical production and vascular injury progression *in vivo* (Hedayati et al., 2017). In addition, regular ingestion of fruit and vegetables can di-

Received 17 February 2020; Accepted 20 April 2020; Published online 30 June 2020
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minish the risk of vascular diseases since many plant constituents contain a phenolic group in their chemical structure, such as flavonoids and phenolic acids, which exhibit antioxidant activity (Hertog et al., 1993; Manach et al., 2004; Yahfoufi et al., 2018). Therefore, studies have been actively conducted to identify plants that can protect endothelial cells against oxidative stress. For instance, Campos et al. (2014) showed that lemongrass polyphenols suppresses oxidative damage in human endothelial cells. Additionally, Wu et al. (2018) showed that the *Polygonum orientale* flower extract can protect human umbilical vein endothelial cells (HUVECs) from hydrogen peroxide (H_2O_2)-triggered oxidative damage by enhancing the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT).

H_2O_2, one of the major components of ROS, can damage the endothelium (Maharjan et al., 2014). H_2O_2 has been widely used to induce oxidative stress in vascular endothelial cells because it can easily penetrate their membranes (Di Marzo et al., 2018). Thus, stimulation with H_2O_2 is a good strategy for investigating vascular endothelial damage.

Vitis vinifera L. is a woody perennial vine plant that usually climbs via tendrils. It is native to Asia Minor, from which it spread to other continents, including Europe (Orhan et al., 2007). The fruit of *V. vinifera* L. is mainly used as food, whereas its seeds and leaves are used as herbal medicine and food supplements. The leaves have a long history of being used as a folk medicine to treat diarrhea, vomiting, wounds, and bleeding in Ayurvedic medicine (Karaman and Kocabas, 2001; Orhan et al., 2009). In previous studies, extracts of *V. vinifera* L. leaves have shown a hepatoprotective effect against carbon tetrachloride-induced acute liver damage and alcohol-induced oxidative damage in rats (Karaman and Kocabas, 2001; Orhan et al., 2007; Pari and Suresh, 2008; Orhan et al., 2009). Recently, Sangiovanni et al. (2019) also showed that *V. vinifera* L. leaf extracts (VE) with the highest quercetin 3-glucuronide content among phenols inhibits inflammation in human keratinocytes. In particular, the leaf extracts were shown to contain higher levels of total phenols and proanthocyanidins than stalk and marc extracts, and exhibited a greater effect on reducing intracellular ROS induced by H_2O_2 in human dermal fibroblasts (Puglisi et al., 2019). Despite these previous studies on *V. vinifera* L. leaf, its role in protecting vascular endothelial cells against oxidative damage has not previously been reported.

Centella asiatica is a traditional medicinal plant found in various tropical regions, including Asia, South Africa, and the Middle East Africa. *C. asiatica* has long been used as a traditional medicine for wound healing and scar management in Asia (Fong et al., 2016). It also has various biological effects, such as anti-inflammatory, antioxidant, neuroprotective, and hepatoprotective activities (Somchit et al., 2004; Zhang et al., 2010; Orhan, 2012; Zhao et al., 2014). Recently, the major triterpenoid compounds of *C. asiatica*, asiaticoside, and asiatic acid were shown to inhibit endothelial hyperpermeability triggered by tumor necrosis factor-α (TNF-α) in human aortic endothelial cells (Fong et al., 2015; Fong et al., 2016). Moreover, madecassoside, another triterpenoid in *C. asiatica*, has been shown to inhibit apoptosis of HUVECs following exposure to H_2O_2-induced oxidative stress (Bian et al., 2012). Therefore, *C. asiatica* might protect vascular endothelial cells against oxidative stress.

Recently, the synergistic effects of phytochemicals have received increasing interest, with the aim of achieving more effective therapies for treating various diseases, including vascular diseases. Because both *V. vinifera* L. leaf and *C. asiatica* contain various active compounds, such as flavonoids, anthocyanidins, and triterpenoids, that exhibit anti-inflammatory and antioxidant effects, the combination of these two extracts may have a synergistic effect. Here, we investigated whether the standardized herbal combination of *V. vinifera* L. leaf extract and *C. asiatica* extract is effective in protecting vascular endothelial cells against oxidative stress induced by H_2O_2.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile and high-performance liquid chromatography (HPLC) grade water were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). H_2O_2 and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Oxygen radical antioxidant capacity (ORAC) activity assay kit and intracellular ROS assay kits were purchased from Cell Biolabs Inc. (San Diego, CA, USA) and Abcam (Cambridge, UK), respectively. The total nitric oxide (NO) assay kit and human vascular endothelial-cadherin (VE-cadherin) enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems (Minneapolis, MN, USA). The *in vitro* vascular permeability assay kit was purchased from Millipore (Bedford, MA, USA).

V. vinifera L. leaf and *C. asiatica* extracts preparation

Dried leaves of *V. vinifera* L. and *C. asiatica* were purchased from an herbal market (Danyang, China). The ethanol extract of *V. vinifera* L. leaf (VE) was obtained by macerating 200 g of dried leaves with 40% ethanol for 4 h. The spent plant material was extracted again using the same solvent. After filtering the extract using Advantec filter paper No. 1 (Advantec, Japan), the combined extracts were concentrated and dried. The ethanol extract of *C. asiatica* (CE) was obtained by macerating 200 g of

dried leaves with 70% ethanol for 3 h and was refluxed twice more. Obtained extracts were combined, filtered, concentrated, and the residual extract was dried. The *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC) was prepared by mixing dried powders of VE and CE at a ratio of 3:1 to produce standardized VCEC.

Calibration of the HPLC method

The analysis of quercetin 3-*O*-β-D-glucuronide (quercetin 3-glucuronide) and asiaticoside was performed with reference to methods previously published (Schneider et al., 2008; Rafamantanana et al., 2009; Benmeziane et al., 2016; da Rocha et al., 2017). Approximately 3.0 mg each of quercetin 3-glucuronide and asiaticoside standard compounds were placed in a 3-mL volumetric flask and dissolved with methanol. Further calibration concentrations were prepared by diluting a standard solution with methanol. The detector response was linear within the range of concentrations injected (quercetin 3-glucuronide: 5～80 μg/mL, asiaticoside: 25～400 μg/mL). The calibration data are listed in Table 1.

HPLC analysis of quercetin 3-glucuronide and asiaticoside

An Agilent HPLC 1200 series system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a quaternary pump, autosampler, and photodiode array detector with a Phenomenex® Luna C18 column (250×4.6 mm, 5 μm) was used for HPLC analysis (Phenomenex Inc., Torrance, CA, USA). For the analysis of quercetin 3-glucuronide, we eluted the extract with solvent A (acetonitrile) and solvent B (1% formic acid in water) in a gradient elution at a flow rate of 0.7 mL/min, as follows: 0～22 min, 83% B; 22～28 min, 83～20% B; 28～33 min, 20% B; 33～35 min, 20～83% B; and 35～40 min, 83% B. The column temperature was kept at 25°C, and the absorbance was detected at 360 nm. For analysis of asiaticoside, we eluted with solvent A (acetonitrile) and solvent B (0.3% phosphoric acid in water) in a gradient elution at a flow rate of 1 mL/min, as follows: 0～5 min, 75% B; 5～20 min, 75～65% B; 20～25 min, 65～25% B; 25～30 min, 25% B; 30～32 min, 25～75% B; and 32～40 min, 75% B. The column temperature was kept at 35°C, and the detection wavelength was 205 nm. The injection volume of all samples was 10 μL.

ORAC assay

ORAC was measured using the OxiSelect™ ORAC assay kit following the manufacturer's instructions. Fluorescence was detected at 480 nm [excitation (ex)] and 520 nm [emission (em)] every 3 min for a total of 180 min using a Victor 3 multilabel plate reader (PerkinElmer, Inc., Norwalk, CT, USA). The area under the curve of each sample was calculated and compared with the Trolox™ (2.5～50 μM) antioxidant standard curve. ORAC values were presented in μM Trolox equivalents per g of VE, CE, and VCEC.

Cell culture

HUVECs were purchased from Lonza (Baltimore, MD, USA). Cells were cultured in M200 (Gibco, Grand Island, NY, USA) supplemented with an low serum growth supplement kit (Gibco) and incubated at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 2 days until cells were confluent. All experiments were performed using cells at passages 3～6.

Cell viability

To measure cell viability, MTT assays were used. MTT assays are based on the ability of living cells to metabolize a water-soluble tetrazolium salt into a water-insoluble formazan crystal. HUVECs (2×10^5 cells/mL) were cultured for 24 h in a 96-well plate and then treated with various concentrations of VE, CE, and VCEC for 24 h. The medium was discarded, and then 5 mg/mL MTT solution was added to each well. After incubation for 3 h at 37°C, the MTT solution was discarded, and 100 μL of dimethyl sulfoxide was added to each well to solubilize formazan. The absorbance was detected at 540 nm with a Victor 3 multilabel plate reader (PerkinElmer, Inc.).

Intracellular ROS production

Intracellular ROS production was measured using the cell-permeable reagent 2',7'-dichlorofluorescein (DCF)-diacetate (DA) (Mendis et al., 2007). HUVECs (3×10^5 cells/mL) were cultured in a 96-well black plate for 24 h and then treated with various concentrations of VE, CE, and VCEC for 30 min, followed by 500 μM H_2O_2 for 4 h. Cells were washed with assay buffer and then incubated with 25 μM 2',7'-dichlorodihydrofluorescein (DCFH)-DA in assay buffer supplemented with 10% fetal bovine serum for 45 min at 37°C. ROS was measured by detecting DCF fluorescence (ex: 485 nm, em: 535 nm) using a Victor 3 multilabel plate reader (PerkinElmer, Inc.).

Table 1. Regression equation, correlation coefficients (R^2), limits of detection, and quantification for high-performance liquid chromatography analysis

Compound	Calibration curve equation, R^2	Limit of detection (μg/mL)	Limit of quantification (μg/mL)
Quercetin 3-glucuronide	y=29.19x+15.05, 0.9992	0.19	0.56
Asiaticoside	y=2.4256x+7.5597, 0.9992	0.39	1.19

NO release

HUVECs (2×10^5 cells/mL) were cultured in a 96-well plate for 24 h. HUVECs were then treated with 37.5 μg/mL of VE, 12.5 μg/mL of CE, and 50 μg/mL of VCEC for 30 min, followed by 500 μM H_2O_2 for 4 h. The amount of NO in culture supernatants was spectrophotometrically analyzed by evaluating the accumulation of nitrate and nitrite using a colorimetric NO assay kit according to the manufacturer's instructions. The absorbance was detected at 540 nm using a Victor 3 multilabel plate reader (PerkinElmer, Inc.).

Transendothelial electrical resistance (TEER) assay

The TEER across the HUVEC monolayer was measured using a Millicell-ERS ohmmeter (Millipore) (Kaneda et al., 2006). HUVECs (0.5 mL) were seeded in the inserts of transwell plates (12-well type, 4×10^5 cells/well), and 1.5 mL of culture medium was added to the receiver plate wells. The culture medium was changed every 2 days. Once cells formed a tight monolayer, cells were treated with 37.5 μg/mL of VE, 12.5 μg/mL of CE, and 50 μg/mL of VCEC for 30 min, followed by 500 μM H_2O_2 for 4 h. TEER ($\Omega\cdot cm^2$) was determined by multiplying the measured electrical resistance (Ω) by the surface area of the membrane (cm^2).

Vascular permeability assay

Vascular permeability was measured using an *in vitro* vascular permeability assay kit according to the manufacturer's instructions. Briefly, 0.2 mL of HUVECs were seeded on the inserts of transwell plates (24-well type, 2×10^5 cells/well), and 0.5 mL of culture medium were added to the receiver plate wells. The culture medium was changed every 2 days, and cells were incubated until a monolayer was formed. Cells were treated with 37.5 μg/mL of VE, 12.5 μg/mL of CE, and 50 μg/mL of VCEC for 30 min, followed by 500 μM H_2O_2 for 4 h. Then, fluorescein isothiocyanate (FITC)-dextran was added to the inserts and allowed to penetrate the monolayers for 20 min. A total of 0.1 mL of the media in each receiver plate well was transferred into a black 96-well plate. The fluorescence intensity was detected at 480 nm (ex) and 520 nm (em) using a Victor 3 multilabel plate reader (PerkinElmer, Inc.).

VE-cadherin expression

HUVECs (3×10^6 cells/mL) were cultured in a 12-well plate for 24 h and then treated with 37.5 μg/mL of VE, 12.5 μg/mL of CE, and 50 μg/mL of VCEC for 30 min, followed by 500 μM H_2O_2 for 4 h. The cells were lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (ATTO, Tokyo, Japan). The levels of VE-cadherin in the cell lysates were detected using a human VE-cadherin ELISA kit following the manufacturer's instructions.

Statistical analysis

The data are expressed as mean±standard deviation (SD). All experiments were performed in triplicate and repeated three times. Statistical significance was analyzed by Student's *t*-test, and $P<0.05$ was considered significant. Statistical tests were conducted using the SPSS standard version 26 (SPSS Inc., Chicago, IL, USA).

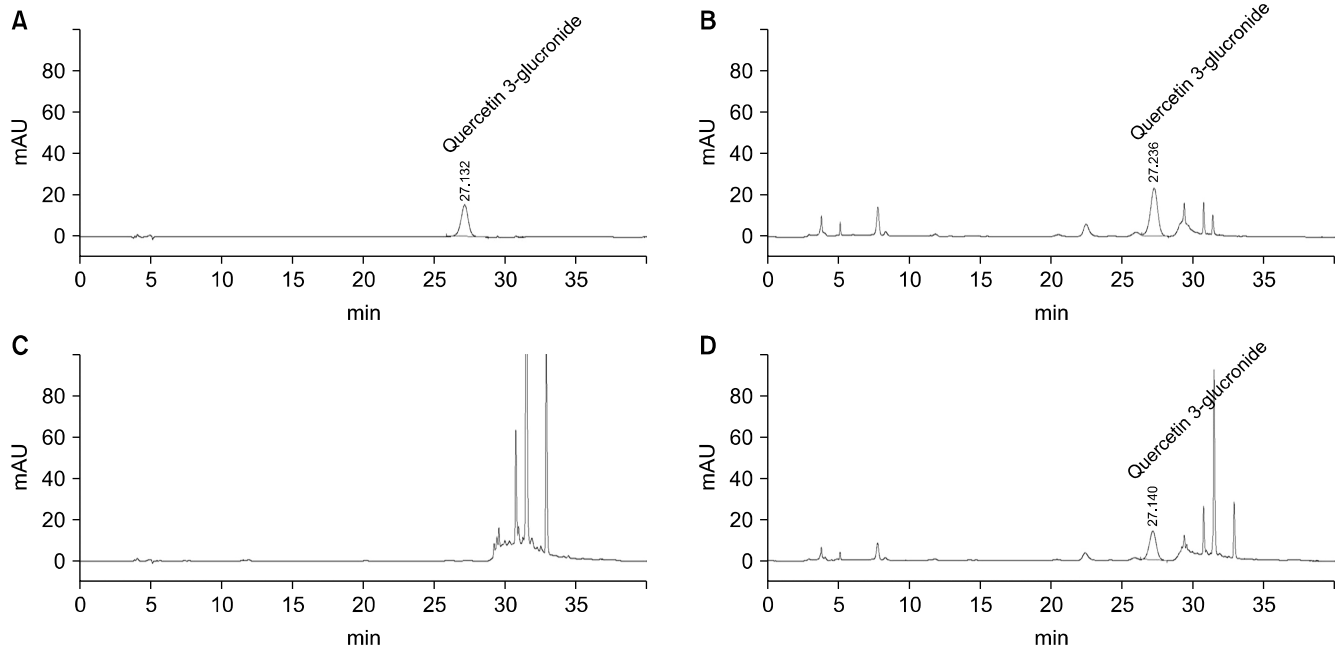

Fig. 1. Representative high-performance liquid chromatography chromatogram at 360 nm: standard solutions of (A) quercetin 3-glucuronide, (B) *Vitis vinifera* L. leaf extract, (C) *Centella asiatica* extract, and (D) *V. vinifera* L. leaf and *C. asiatica* extract combination.

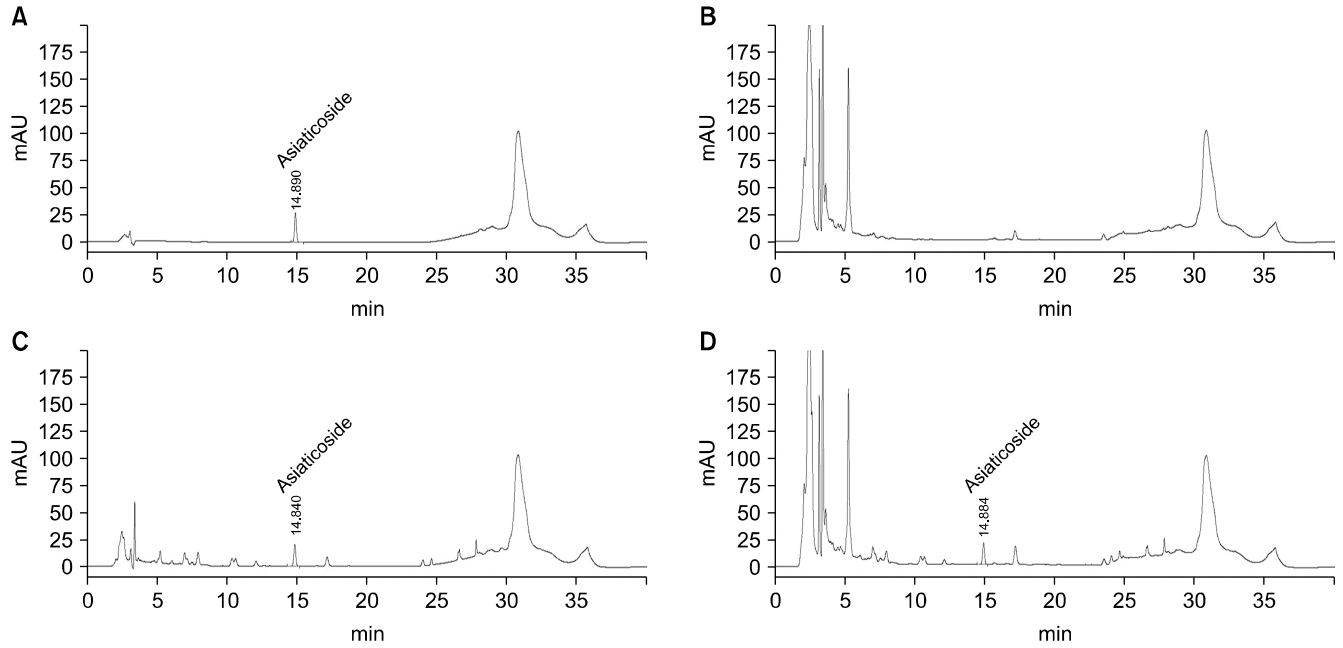

Fig. 2. Representative high-performance liquid chromatography chromatogram at 205 nm: standard solutions of (A) asiaticoside, (B) *Vitis vinifera* L. leaf extract, (C) *Centella asiatica* extract, and (D) *V. vinifera* L. leaf and *C. asiatica* extract combination.

RESULTS

HPLC analysis of VCEC

Based on the absorption profile and retention time, quercetin 3-glucuronide and asiaticoside were identified as the major compounds in VCEC. Fig. 1A and 2A show the HPLC chromatograms of quercetin 3-glucuronide and asiaticoside standards for peak comparison with the chromatogram of VE (Fig. 1B and 2B), CE (Fig. 1C and 2C), and VCEC (Fig. 1D and 2D). According to the HPLC chromatograms, quercetin 3-glucuronide was detected at 360 nm with a retention time of 26.9 min, and asiaticoside was detected at 205 nm with a retention time of 14.9 min. The results confirmed that VE and VCEC contained 11.4±0.16 and 8.5±0.12 mg/g of quercetin 3-glucuronide, respectively, and CE and VCEC contained 95.6±0.22 and 24.9±0.17 mg/g of asiaticoside, respectively (Table 2).

Table 2. Contents of quercetin 3-glucuronide and asiaticoside in VCEC (unit: mg/g)

Content	Compound	
	Quercetin 3-glucuronide	Asiaticoside
VE	11.4±0.16	ND
CE	ND	95.6±0.22
VCEC	8.5±0.12	24.9±0.17

Data are expressed as mean±SD of three replicate experiments.
ND: not detected.
VE, *Vitis vinifera* L. leaf extract; CE, *Centella asiatica* extract; VCEC, *V. vinifera* L. leaf and *C. asiatica* extract combination.

Antioxidant activity of VCEC

To confirm the antioxidant activity of VCEC and individual extracts of VE and CE, ORAC assays were conducted using Trolox™ as the standard. VE and CE showed similar antioxidant activities (Fig. 3). Remarkably, VCEC demonstrated ~1.7-fold higher antioxidant activity than VE or CE alone ($P<0.01$), suggesting that VE and CE have a synergistic effect on antioxidant activity.

Effect on HUVEC viability

We investigated the effect of VCEC on the vascular endothelium using HUVECs, a classical model system for studying endothelial function. We treated cells with VE and CE at the same concentration as contained in VCEC to evaluate whether VC and CE show synergistic effects on endothelial function compared with each compound alone. First, we examined the effects of VE, CE, and VCEC on HUVEC viability to investigate potential cell cytotoxicity. Concentrations of up to 37.5 μg/mL of VE, 12.5 μg/mL of CE, and 50 μg/mL of VCEC (37.5 μg/mL of VE+12.5 μg/mL of CE) did not affect the viability of HUVECs, as determined by the MTT viability assays (Fig. 4).

Effect on intracellular ROS production

To examine the effect of VCEC on ROS production, we measured intracellular ROS by using a DCFH-DA fluorescent probe. The fluorescence intensity of cells exposed to H_2O_2 alone dramatically increased to ~7.6-fold compared with the control group ($P<0.001$); however, pretreatment with VCEC (6.25, 12.5, 25, or 50 μg/mL) significantly attenuated the increase in fluorescence intensi-

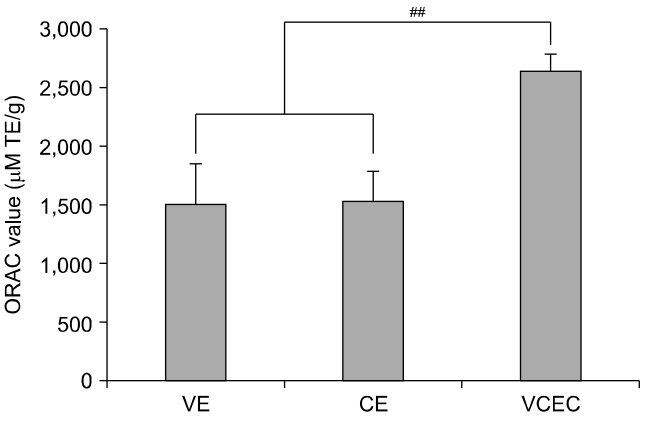

Fig. 3. Antioxidant activity of *Vitis vinifera* L. leaf extract (VE), *Centella asiatica* extract (CE), and *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC) in oxygen radical antioxidant capacity (ORAC) assays. The antioxidant activities of VE, CE, or VCEC were evaluated using ORAC assays. Total antioxidant activity is expressed as μM Trolox equivalents (TE)/g. Values are shown as the mean±SD (n=3 with three replicates in each experiment). $^{\#\#}P<0.01$ compared with the VE or CE group.

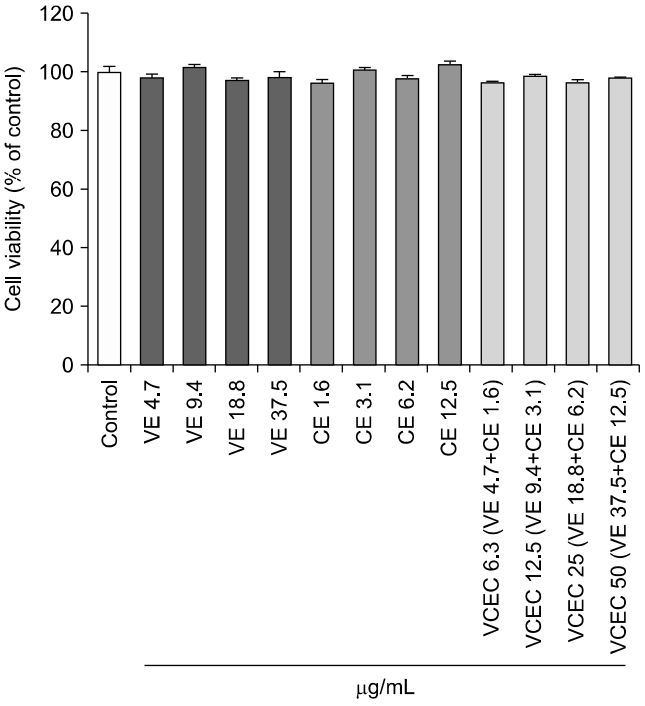

Fig. 4. Effects of *Vitis vinifera* L. leaf extract (VE), *Centella asiatica* extract (CE), and *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC) on the viability of HUVECs. Cells were treated with various concentrations of VE, CE, or VCEC for 24 h. Cell viability was determined by the thiazolyl blue tetrazolium bromide assays. The results are expressed as a percentage of the control. Values are the mean±SD (n=3 with three replicates in each experiment).

ty induced by H_2O_2 ($P<0.001$) in a dose-dependent manner (Fig. 5). In particular, 25 and 50 μg/mL of VCEC decreased the amount of ROS to a level similar to that of the control group. Pretreatment with VE (4.69, 9.38, 18.75, or 37.5 μg/mL) or CE (3.12, 6.25, or 12.5 μg/mL) also significantly inhibited ROS production, but pretreatment with VCEC induced a greater inhibitory effect compared with that for VE or CE alone.

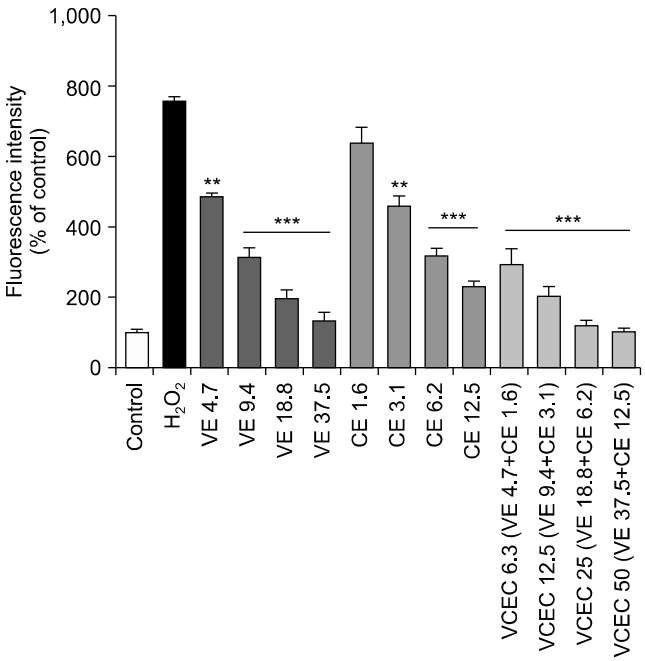

Fig. 5. Effects of *Vitis vinifera* L. leaf extract (VE), *Centella asiatica* extract (CE), and *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC) on the intracellular reactive oxygen species (ROS) production in human umbilical vein endothelial cells in conditions of oxidative stress induced by H_2O_2. Cells were treated with various concentrations of VE, CE, and VCEC for 30 min before exposure to 500 μM H_2O_2 for 4 h. ROS production was determined using 25 μM of 2',7'-dichlorofluorescein diacetate. The results are expressed as a percentage of the control. Values are shown as the mean±SD (n=3 with three replicates in each experiment). $^{**}P<0.01$ and $^{***}P<0.001$ compared with the H_2O_2-alone group.

Effect on NO level

To examine the effect of VCEC on NO levels, we measured the total NO in the culture supernatants. Exposure to H_2O_2 for 4 h considerably reduced NO release to 86.5 ±3.7% compared with the control group ($P<0.05$) (Fig. 6). Pretreatment of cells with 37.5 μg/mL of VE and 50 μg/mL of VCEC (37.5 μg/mL of VE+12.5 μg/mL of CE) significantly suppressed the decrease in NO release induced by exposure to H_2O_2, whereas NO release was not impacted by 12.5 μg/mL of CE.

Effect on HUVEC TEER

We further estimated the ability of VCEC to preserve the endothelial monolayer barrier function by using TEER measurements and permeability assays. First, TEER in the HUVEC monolayer was measured using a Millicell-ERS ohmmeter during H_2O_2 exposure. H_2O_2 substantially decreased TEER to 61±3% compared with the control group (Fig. 7), indicative of damage to the endothelial barrier. Pretreatment of the HUVEC monolayers with 37.5 μg/mL of VE and 50 μg/mL of VCEC (37.5 μg/mL

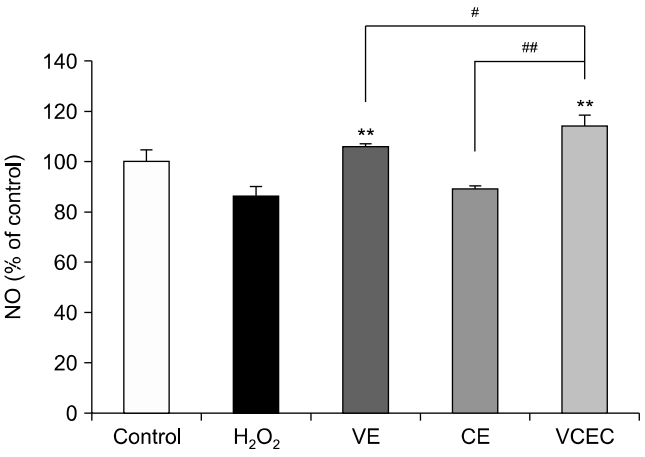

Fig. 6. Effects of *Vitis vinifera* L. leaf extract (VE), *Centella asiatica* extract (CE), and *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC) on nitric oxide production in human umbilical vein endothelial cells in conditions of oxidative stress induced by H_2O_2. Cells were treated with 37.5 μg/mL of VE, 12.5 μg/mL of CE, or 50 μg/mL of VCEC (37.5 μg/mL of VE+12.5 μg/mL of CE) for 30 min before exposure to 500 μM H_2O_2 for 4 h. Total nitrite concentrations in culture supernatants were measured using the Griess reagent. The results are expressed as a percentage of the control. Values are presented as mean±SD (n=3 with three replicates in each experiment). $^{**}P<0.01$ compared with the H_2O_2-alone group; $^{\#}P<0.05$ or $^{\#\#}P<0.01$ compared with the VE or CE group.

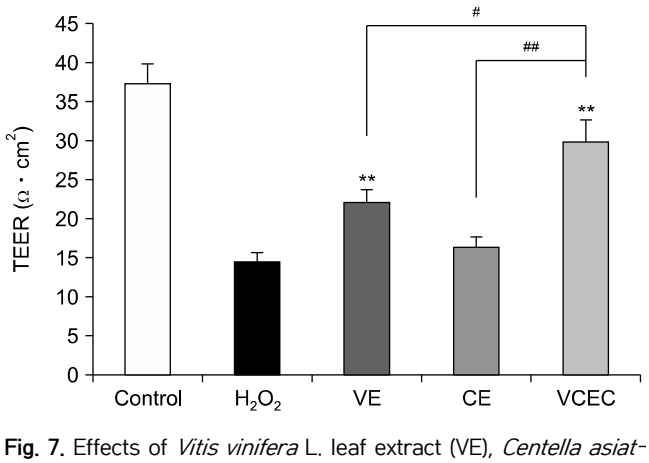

Fig. 7. Effects of *Vitis vinifera* L. leaf extract (VE), *Centella asiatica* extract (CE), and *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC) on the transendothelial electrical resistance (TEER) of human umbilical vein endothelial cell (HUVEC) monolayers in conditions of oxidative stress induced by H_2O_2. Cells were treated with 37.5 μg/mL of VE, 12.5 μg/mL of CE, or 50 μg/mL of VCEC (37.5 μg/mL of VE+12.5 μg/mL of CE) for 30 min before exposure to 500 μM H_2O_2 for 4 h. The TEER of HUVEC monolayers was detected using a Millicell-ERS ohmmeter. Values represent the mean±SD (n=3 with three replicates in each experiment). $^{**}P<0.01$ compared with the H_2O_2-alone group; $^{\#}P<0.05$ or $^{\#\#}P<0.01$ compared with the VE or CE group.

of VE+12.5 μg/mL of CE) for 30 min prior to H_2O_2 exposure protected the monolayer integrity against H_2O_2-induced endothelial barrier dysfunction, whereas 12.5 μg/mL CE was ineffective. In addition, treatment of the monolayer with VCEC significantly attenuated the reduction in TEER to a greater extent than treatment with VE alone.

Effect on HUVEC monolayer permeability

In addition to measuring TEER, permeability assays were conducted to investigate the integrity of the HUVEC monolayer. We used an *in vitro* vascular permeability assay kit to measure the accumulation of FITC-conjugated dextran passing through the HUVEC monolayer. H_2O_2 significantly increased permeability to 218.3±16.9% compared with the control group, and pretreatment with 37.5 μg/mL of VE, 12.5 μg/mL of CE, and 50 μg/mL VCEC (37.5 μg/mL of VE+12.5 μg/mL of CE) prominently abolished the H_2O_2-induced hyperpermeability (Fig. 8). Treatment of the monolayer with VCEC showed a significantly greater inhibitory effect that treatment with VE or CE alone. To confirm the unexpected synergistic effect of VE and CE on HUVEC permeability, we used Colby's equation (Colby, 1967) to determine the benefit of combining VE and CE. This equation has been used to evaluate the benefit of other herbal extract mixtures (Yimam et al., 2016a). The observed value (77.8%) was greater than the expected hypothetical value (74.8%), indicating the existence of a synergistic effect of the two extracts (Table 3).

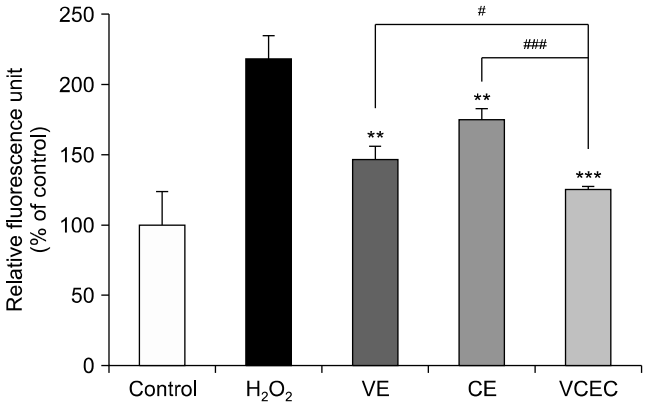

Fig. 8. Effects of *Vitis vinifera* L. leaf extract (VE), *Centella asiatica* extract (CE), and *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC) on the monolayer permeability of human umbilical vein endothelial cells in conditions of oxidative stress conditions induced by H_2O_2. Cells were treated with 37.5 μg/mL of VE, 12.5 μg/mL of CE, or 50 μg/mL of VCEC (37.5 μg/mL of VE+12.5 μg/mL of CE) for 30 min before exposure to 500 μM H_2O_2 for 4 h. The fluorescent intensities of fluorescein isothiocyanate-dextran in receiver wells were quantified at an excitation/emission wavelength of 485/535 nm. The results are expressed as a percentage of the control. Values are the mean±SD (n=3 with three replicates in each experiment). $^{**}P<0.01$ or $^{***}P<0.001$ compared with the H_2O_2-alone group; $^{\#}P<0.05$ or $^{\#\#\#}P<0.001$ compared with the VE or CE group.

Effect on VE-cadherin expression

To elucidate the regulatory mechanism of VCEC on endothelial barrier function, the expression of VE-cadherin, a major component of endothelial adherens junctions, was assessed by ELISA. H_2O_2 decreased the expression of

Table 3. Unexpected synergistic effect of *Vitis vinifera* L. and *Centella asiatica* on vascular permeability

Composition	Material	Dose (μg/mL)	Percent inhibition
VCEC	VE	35.5	60.6 (*x*)
	CE	12.5	36.1 (*y*)
	Expected[1)]	50	74.8
	Observed[2)]	50	77.8

Colby's equation=$x+y-(xy)/100$.
[1)]Calculated value according to Colby's method.
[2)]Data observed when a composition was treated at 50 μg/mL, when observed≥expected=unexpected synergy.
VCEC, *V. vinifera* L. leaf and *C. asiatica* extract combination; VE, *V. vinifera* L. leaf extract; CE, *C. asiatica* extract.

VE-cadherin to 77.8% of the control (Fig. 9). However, pretreatment with 37.5 μg/mL of VE, 12.5 μg/mL of CE, and 50 μg/mL of VCEC (37.5 μg/mL of VE+12.5 μg/mL of CE) significantly abolished the H_2O_2-induced reduction in VE-cadherin expression. In particular, there was a greater inhibitory effect for treatment with VCEC compared with VE or CE alone.

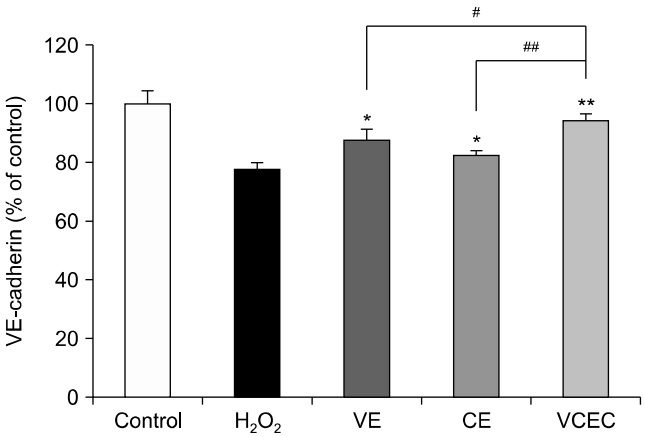

Fig. 9. Effects of *Vitis vinifera* L. leaf extract (VE), *Centella asiatica* extract (CE), and *V. vinifera* L. leaf and *C. asiatica* extract combination (VCEC) on vascular endothelial-cadherin (VE-cadherin) expression in human umbilical vein endothelial cells in conditions of oxidative stress induced by H_2O_2. Cells were treated with 37.5 μg/mL of VE, 12.5 μg/mL of CE, or 50 μg/mL of VCEC (37.5 μg/mL of VE+12.5 μg/mL of CE) for 30 min before exposure to 500 μM H_2O_2 for 4 h. VE-cadherin concentrations were determined with an ELISA. The results are expressed as a percentage of the control. Values are shown as the mean±SD (n=3 with three replicates in each experiment). $^{*}P<0.05$ or $^{**}P<0.01$ compared with the H_2O_2-alone group; $^{\#}P<0.05$ or $^{\#\#}P<0.01$ compared with the VE or CE group.

DISCUSSION

Vascular disease, a disorder of the blood vessels, is one of the most prominent chronic diseases, and develops gradually over a long period of time (Ellulu et al., 2016). Endothelial damage induced by oxidative stress related to obesity, hyperlipidemia, and aging is considered to be an early biomarker in the pathogenesis of several vascular diseases (Pandian et al., 2005; Banerjee et al., 2019). A previous study demonstrated that high levels of oxidative stress are related to a remarkable increase in endothelial cell cytotoxicity (Hermann et al., 1997). Therefore, there is now a substantial amount of research being conducted on new antioxidant agents that can reduce the oxidative stress of blood vessels. Recently, Mo et al. (2017) showed that scutellarin, a major active compound in *Erigeron breviscapus*, ameliorated hyperlipidemia and oxidative injury in rats fed a high-fat diet. Moreover, Gasparotto et al. (2019) showed that *Echinodorus grandiflorus* extracts reduce atherosclerotic lesions in rabbits receiving a cholesterol-rich diet by decreasing lipid and protein oxidation. Thus, the discovery of new antioxidant agents that reduce oxidative stress in the endothelium is a useful strategy for decreasing the risk of vascular disease (Widlansky et al., 2003).

To develop a new herbal combination, we investigated the synergistic effect of VE and CE on the protection of endothelial cells against oxidative stress. First, we examined the antioxidant activities of VE, CE, and VCEC using ORAC assays. VCEC exhibited a significantly greater antioxidant capacity than VE or CE alone. Generally, the antioxidant activities of herbs are mainly associated with their phenolic and flavonoid compounds (Zheng and Wang, 2001). *V. vinifera* L. leaves contain various flavonoids, such as quercetin 3-glucuronide, quercetin 3-glucoside, kaempferol 3-glucoside, and cyanidin 3-glucoside (Sangiovanni et al., 2019). In addition, *C. asiatica* contains a variety of triterpenoids, such as asiaticoside, asiatic acid, madecassoside, and madecassic acid (Hashim et al., 2011). Because using different compounds together may affect their properties, mixtures of plants can show synergistic effects. For instance, rosemary and sage extracts exhibit a synergistic effect on antioxidant activity (Irwandi et al., 2000), and *Salvia miltiorrhiza* and *Dalbergia odorifera* extracts synergistically attenuate rat myocardial ischemia/reperfusion injury (Mu et al., 2017). Moreover, when we investigated H_2O_2-induced intracellular ROS production in HUVECs, pretreatment with VCEC inhibited ROS production to a greater extent than VE or CE alone. As mentioned above, oxidative stress induced by ROS induces endothelial damage via oxidation of the cell membrane, nucleotides, and proteins. Furthermore, ROS can trigger the generation of inflammatory cytokines, such as TNF-α and interleukin-8, as well as cell adhesion molecules, which induce binding of monocytes to endothelial cells (DeForge et al., 1993; Yamada, 2001). Therefore, the inhibitory effect of VCEC on ROS production is critical for protection of the endothelium against oxidative stress.

NO is one of the most critical regulators of endothelial function that is released by endothelial cells. NO is a vas-

orelaxant that plays a beneficial role in maintaining blood pressure and cardiovascular physiology (Dimmeler and Zeiher, 1999). Decreased levels of endothelium-derived NO are associated with pathophysiological modifications that occur in several vascular diseases (Drexler and Hornig, 1999). In previous studies, H_2O_2 has been shown to inhibit NO production via reducing endothelial NO synthase activity and to increase hyperpermeability of endothelial cells (Boulden et al., 2006; Yin et al., 2014). Consistent with this finding, our results showed that H_2O_2 significantly reduced NO production in HUVECs. However, preincubation with VCEC significantly attenuated the H_2O_2-induced decrease in NO generation to a greater degree than preincubation with VE or CE. These findings suggest that VE may exert a synergistic effect with CE on NO production.

In addition, we evaluated the ability of VCEC to protect the endothelial barrier function of HUVEC monolayers against H_2O_2-induced oxidative stress. The endothelial cell monolayer barrier is critical for maintaining vascular homeostasis, and forming a gateway between the vascular lumen and vascular wall (Rodrigues and Granger, 2015). Various factors can disturb the structure and function of the endothelial barrier and ultimately cause numerous vascular diseases. One of these factors is oxidative stress; during oxidative stress, ROS can induce barrier leakage by upregulating vascular endothelial growth factor (VEGF) and disrupting the integrity of tight junctions, which subsequently increases permeability to macromolecules, fluids, and inflammatory cells (Chua et al., 1998; Zhao and Davis, 1998). In this study, we used TEER measurements and permeability assays to estimate the integrity of the endothelial monolayer in HUVECs. If the integrity of the monolayer is unstable, membrane electrical resistance is low, and paracellular transport of FITC-dextran across the monolayer will increase. Our data indicated that H_2O_2 dramatically decreased the TEER value and increased the endothelial permeability of FITC-dextran. This indicates that the decreased TEER value of the monolayer reflects the increase in monolayer permeability. Pretreatment of the HUVEC monolayer with VE, CE, or VCEC substantially inhibited the decrease in TEER value induced by H_2O_2; however, inhibition by CE did not reach statistical significance. Furthermore, the increased FITC-dextran permeability stimulated by H_2O_2 was significantly reduced by the three treatments, in the following order of magnitude: VCEC>VE>CE. In particular, unexpected synergy was observed for VCEC in the permeability assay when calculated by Colby's equation, which has been previously used to determine the benefit of blending other herbal extracts (Yimam et al., 2016b). This protective effect of VCEC on barrier integrity might be related to its ability to inhibit ROS production and increase NO. Furthermore, VCEC upregulated VE-cadherin in conditions of oxidative stress. VE-cadherin is a major molecule involved in adhesion at endothelial adherens junctions and thus regulates paracellular permeability (Sarelius and Glading, 2015). Various inflammatory mediators, including cytokines and oxidative stress, reduce VE-cadherin expression in vascular endothelial cells and, consequently, increase endothelial cell permeability (Alexander et al., 2000; Hofmann et al., 2002). We showed that VCEC inhibited the decrease in VE-cadherin expression induced by H_2O_2. This result suggests that VCEC protects the endothelial barrier function by regulating adhesion molecules. However, other signaling factors may also be associated with the activity of VCEC.

The mitogen-activated protein kinase (MAPK) family of proteins, which includes p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun-NH_2-terminal kinase, is known to regulate vascular permeability (Bogatcheva et al., 2003). Specifically, it has been reported that H_2O_2 triggers an increase in HUVEC solute permeability through disorganizing tight junction proteins, such as occludin and zonula occludens (ZO)-1, via activating ERK1/2 and/or p38 MAPK pathways (Kevil et al., 2000; Kevil et al., 2001). Additionally, H_2O_2 induces vascular permeability by upregulating VEGF, a vascular permeability factor and an endothelial cell-specific mitogen (Chua et al., 1998; Lee et al., 2006). In recent studies, VE has been shown to counteract the release of VEGF induced by H_2O_2 in human keratinocytes, and stem extracts have been shown to reduces levels of VEGF in human endothelial cells (Stagos et al., 2014; Sangiovanni et al., 2019). In another study, the major compound of VE, quercetin 3-glucuronide, was shown to inhibit ROS generation and lipid peroxidation by increasing the activities of antioxidant enzymes, such as SOD and CAT, by upregulating heme oxygenase-1 via the transcription factors nuclear factor E2-related factor 2 and activator protein-1 (Lee et al., 2017). In the case of *C. asiatica*, the major active compounds asiaticoside and asiatic acid protect against TNF-α-induced endothelial barrier dysfunction by abrogating the structural reorganization of occludin, ZO-1, and adherens junction proteins, such as VE-cadherin and β-catenin (Fong et al., 2015; Fong et al., 2019). Furthermore, madecassoside, another active constituent, inhibits p38 MAPK activity induced by H_2O_2 in HUVECs (Bian et al., 2012). Given these findings, it is possible that various compounds contained in the standardized blend of VCEC may have synergistic effects for protecting endothelial barrier function against oxidative damage, acting via multiple mechanisms of action. However, further research is needed to elucidate the specific compounds that contribute to the synergistic effects on antioxidant activity and endothelial barrier function.

In conclusion, our study has demonstrated for the first time that combining extracts from the leaves of *V. vinifera*

and *C. asiatica* had a synergistic effect on protecting the endothelium against oxidative stress. Compared with VE or CE alone, VCEC shows a stronger protective effect on endothelial barrier function owing to its antioxidant activity and regulation of NO and VE-cadherin levels. Therefore, VCEC may potentially be considered a useful therapeutic for the treatment of vascular diseases associated with oxidative stress. However, *in vivo* studies and clinical trials are required to prove the efficacy of VCEC in reducing the risk of vascular diseases.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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