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Cytoprotective effect of *Vernonia cinerea* Less. extract on human umbilical vein endothelial cells against nicotine toxicity

Roongtawan Supabphol[1]*, Rith Wattanachaiyingcharoen[2], Narisa Kamkaen[3] and Athikom Supabphol[4]

[1]Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand.
[2]Faculty of Pharmacy, Srinakharinwirot University, Ongkharak, Nakorn Nayok, Thailand.
[3]Faculty of Science and Technology, Suan Dusit Rajabhat University, Dusit, Bangkok, Thailand.
[4]Department of Surgery, Faculty of Medicine, HRH Princess Maha Chakri Sirindhorn Medical Center (MSMC), Srinakharinwirot University, Ongkharak, Nakorn Nayok, Thailand.

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Every part of *Vernonia cinerea* Less. has been reported for medicinal uses as they all have various therapeutic values with many kinds of pure compounds isolated. The aim of the present study is to investigate the cytoprotective effect and mechanisms of a whole plant extract on human umbilical vein endothelial cells (HUVECs) from nicotine toxicity. Cytotoxic ability of nicotine and *V. cinerea* extract to HUVECs were determined by proliferation assay using the 3-(4,5-dimethylthia-zol,2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. After that, the cytoprotective effect of *V. cinerea* was assessed by examining the presence of vacuole-like structures in cells exposed to 5 or 7.5 mM nicotine with and without *V. cinerea* water extract and stained them with crystal violet. Reverse transcription-polymerase chain reaction (RT-PCR) was used to confirm the mRNA levels of genes involved in intracellular antioxidant system. Although 0.1 to 5 mM nicotine showed no toxic effect on HUVECs during 7 days treatment, abnormal features, that is, the vacuole-like structures were found in the cytoplasm of exposed HUVECs. *V. cinerea* water extract of 100, 500 and 1,000 µg/ml mixed with 5 mM nicotine reduced the numbers of cells containing vacuole-like structures in the cytoplasm of HUVECs with the dose- and time-dependent fashion. The mRNA of catalase and catalase activity in HUVECs exposed to 5 mM nicotine was significantly down-regulated, but recovered when the cells were treated with *V. cinerea* extract. *V. cinerea* extract could be useful in protecting endothelial cells from nicotine toxicity possibly via intracellular antioxidant mechanism, catalase.

Key words: *Vernonia cinerea,* cytoprotective effect, nicotine toxicity, catalase.

INTRODUCTION

Vernonia cinerea Less. (Asteraceae) is an annual herb in the tropical regions mainly widespread in South-East Asia. Virtually every part of this plant has been reported for medicinal uses as they all have various therapeutic values. Many kinds of pure compounds isolated are expected to have medical relevance (Chen et al., 2006; Zhu et al., 2009). An alcoholic extract from the flower and whole plant of *V. cinerea* have been reported to possess an anti-inflammatory and antipyretic effect (Mazumder et al., 2003; Iwalewa et al., 2003). The protective effect of

*Corresponding author. E-mail: roongs@swu.ac.th. Tel: +6626495375

V. cinerea to kidney showed its high potential for medical use. The alcoholic extract of *V. cinerea* shows promising nephrocurative activity, whereas ethylacetate extract possesses significant nephroprotective activity to the cisplatin-induced renal toxicity in rat. These extracts might be the source of therapeutic compound useful in treating renal injury (Adikay and Bharath, 2006).

A radioprotective effect occurring via antioxidant status of *V. cinerea* extract was found in balb/c mice irradiated by gamma-ray. DNA and tissue damages including the solid tumor decrease, indicating the possibility that some compound present in the extract may be used as an adjuvant herbal medicine to revert the side effects occurring during radiotherapy (Pratheeshkumar and Kuttan, 2011a). Methanolic extract of *V. cinerea* given intraperitoneally also shows a significant chemoprotective effect during cyclophosphamide treatment, indicating the high possibility of using it as an adjuvant during chemotherapy (Pratheeshkumar and Kuttan, 2010). The antioxidative status of ethanolic extract of *V. cinerea* scavenges both hydroxyl and superoxide radicals, including significant inhibition of lipid peroxidation. The administration of *V. cinerea* to mice significantly increases the levels of catalase, superoxide dismutase, glutathione, glutathione peroxidase and glutathione-S transferase in blood and liver (Pratheeshkumar and Kuttan, 2009). Two novel sesquiterpene lactones, vernolide-A, and vernolide-B, isolated from ethanolic extract of stem of *V. cinerea* show a potent cytotoxicity and antimetastatic action to cancer cells (Kuo et al., 2003; Pratheeshkumar and Kuttan, 2011b). The relationship between antioxidative potency and anticancer activity was not mentioned.

The recent application for smoking cessation is another medical benefit widely mentioned. Wongwiwatthananukit et al. (2009) reported the high possibility of using *V. cinerea* extracts as an alternative therapeutic target with low cost for the treatment of tobacco dependence. The action of *V. cinerea* extract is due to the generation of unpleasant taste and smell of cigarette smoke which then reduces the craving. *V. cinerea* extract supplementation was currently studied in conjunction with exercise to reduce smoking rate by modifying the levels of oxidative stress and beta-endorphine (Leelarungrayub et al., 2010).

A pharmaceutical product for smoking cessation based on *V. cinerea* extract has been launched into the market. Because of the oral administration, the extract can be absorbed through the gastrointestinal tract and might have some effect on the endothelial cells. The present paper attempted to elucidate the cytoprotective effect of *V. cinerea* extract to nicotine toxicity. The oxidative effect of nicotine was investigated using androgen biomarkers of redox status (Tinti and Soory, 2012). Superoxide anion generation, lipid peroxidation and oxidized glutathione levels were increased significantly in rat, and reduced glutathione level, activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX),glutathione reductase (GSR) and glutathione-s-transferase were decreased significantly in rat with the increasing dose and duration of nicotine treatment (Das et al., 2012). In our study, mRNA expression of key genes regulating intracellular antioxidant capacity, e.g., superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase was assessed for a protective mechanism. The toxicity of *V. cinerea* water extract to human umbilical vein endothelial cells was also studied to confirm the safety of the *V. cinerea* extract.

MATERIALS AND METHODS

Plant materials

Aerial parts of *V. cinerea* were collected from a local garden at Srinakharinwirot University, Ongkharak campus in March 2007 which has been grown without spraying any insecticide or herbicides. Plant was authenticated by comparison with the herbarium specimen at the Princess Sirindhorn Plant Herbarium, Bangkok, Thailand. The plant was washed by tap water and air-dried at room temperature for 7 days.

Crude extract

V. cinerea extract was obtained by maceration. Briefly, the dried plants were grounded into powder and then macerated with purified water and sonicated for 2 h. The filtrate was collected and filtrated under vacuum. The solvent was then removed under vacuum by using freeze dryer. The dried extracts were stored at -20°C until used.

Cell culture

HUVECs was a generous gift from Armed Forces Research Institute of Medical Sciences, Thailand, and cultured as described elsewhere (Supabphol and Supabphol, 2013). The endothelial cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin sulfate (100 µg/ml), heparin (16 U/ml), endothelial cell growth supplement (75 µg/ml), and 2 mM glutamine. Cells were incubated at 37°C in a humidified air with 5% CO_2 condition and subcultured by trypsinization with 0.05% trypsin-0.02% EDTA when 90% confluent monolayers were reached. All chemicals for cell culture were purchased from Gibco BRL.

Cytotoxic assay

Proliferation assay was utilized to determine the survival rate of the endothelial cells by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye as previously described (Supabphol et al., 2009). HUVECs were plated into 24-well tissue culture plate in a density of 3-4 × 10^4 cells/well in a final volume of 1,000 µl. After 90% confluecy, the cells were treated with varying doses of nicotine (0 to 25 mM) or *V. cinerea* extract (0 to 1,000 µg/ml) in complete media for 1, 4 and 7 days. The medium was replaced by fresh medium with MTT dye 0.5 mg/ml medium and the cells were further incubated for 4 h at 37°C until the formation of purple formazan crystals was completed. The crystals were

Table 1. Primers used for RT-PCR analysis.

Gene	Primer sequence (5' – 3')	Size (bp)
Superoxide dismutase	GAGACTTGGGCAATGTGACTG TTACACCACAAGCCAAACGA	201
Catalase	AGTTCGGTTCTCCACTGTTGC CTTGGGTCGAAGGCTATCTGT	681
Glutathione reductase	CTTGCGTGAATGTTGGATGT GACCTCTATTGTGGGCTTGG	257
Glutathione peroxidase	GCCGTGCTGATTGAGAATG AGGTAGGCGAAGACAGGATG	269
β-actin	AGAGCTACGAGCTGCCTGAC ACATTGTGAACTTTGGGGGA	622

solubilized with acidified isopropanol and the absorbance was quantified by spectrophotometry at 570 nm (Bio-Rad 550). Percent survival was plotted against control (untreated) group. Each sample was assayed in triplicate. All chemicals for cell viability assay were obtained from Sigma.

Cytoprotective test

HUVECs were seeded in 24-well culture plate in complete DMEM supplemented with 10% heat-inactivated FBS, endothelial cell growth supplement, glutamine, and antibiotics overnight. After 90% confluent, the medium was replaced by fresh medium containing 5 or 7.5 mM nicotine with or without various concentrations of *V. cinerea* extract (0 to 1,000 µg/ml). After, 24, 48 and 72 h of these treatments, cells were further stained by 0.5% crystal violet (Merck) and the vacuole-like structures were observed and vacuole-containing cells were counted under a microscope (Olympus BX550). In each well, cell count was made on five microscopic fields with a magnification of ×100. On average, 100 to 120 cells were tracked per field and counted as the cells with and without cytoplasmic vacuoles. Each experiment was assayed in triplicate, completely independent from each other.

RNA extraction and RT-PCR

Total RNA was isolated from treated and non-treated HUVECs using Trizol reagent (Invitrogen). The purity and concentration of the isolated RNA were assessed by measuring the absorption at 260 and 280 nm. Same amounts of RNA from each sample were mixed in a 20 µl reaction mixture and single-stranded complementary DNA (cDNA) was synthesized by SuperScript RT kit (Invitrogen) according to the manufacturer's instructions.

The primers in Table 1 were used to amplify the target genes using i-Taq kit (iNtRON Biotechnology). Amplification products obtained by PCR (Bio-Rad C1000) were electrophoretically separated on a 2% agarose gel, stained by 2% ethidium bromide and photograped. Each sample was assayed in triplicate.

Catalase activity assay

Catalase activity was performed using the ability of catalase to decompose H_2O_2 molecules as previously described (Tangjitjaroenkun et al., 2012).

Statistical analysis

Data are expressed as the mean of three independent experiments ± standard deviation of control. Determination of statistical significance was performed by analysis of variance using SPSS IBM Singapore Pte Ltd (Registration No.1975-01566-C). Statistical significance was considered when $p < 0.05$. Statistical comparisons between groups were performed using the Student's t-test.

RESULTS

Effect of nicotine on HUVEC proliferation

To establish the toxic doses of nicotine to HUVECs, proliferation or cell viability assays was performed by using MTT dye to differentiate the viable and dead cells. The incubation with nicotine lower than 5 mM up to 7 days showed no significant reduction in the viability of HUVECs. In contrast, data in Figure 1 shows that nicotine effectively blocked HUVEC proliferation at concentrations greater than 5 mM with both dose- and time-dependent fashion. In addition, HUVECs exposed to nicotine from 5 to 25 mM showed vacuole-like structures in the cyto-plasm of living cells (data not shown). Vacuole-like structure seemed to make cells swollen and looked enlarged. The degree of this unusual feature occurred in dose- and time-dependent manner. The nicotine concen-tration at 5 mM was selected for further experiments because it was the lowest concentration causing vacuole-like structure with no significant inhibition of cell growth.

Effect of *V. cinerea* extract on HUVEC proliferation

Effect of *V. cinerea* extract to HUVEC proliferation was performed by the same method as nicotine using MTT

Table 2. Effect of *V. cinerea* extract to vacuole-like structure in HUVEC cytoplasm. Endothelial cells were treated with 5 mM nicotine and *V. cinerea* extract at the concentration of 0, 100, 500 and 1,000 µg/ml for 24, 48, and 72 h.

[Nicotine] (mM)	[VC extract] (µg/ml)	Percentage of normal endothelial cells (no vacuole-like structure in cytoplasm) with different exposure time to *V. cinerea* extract		
		24 h	48 h	72 h
0	0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
5	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5	100	96.1 ± 0.4*	96.1 ± 0.4*	100.0 ± 0.0*
5	500	100.0 ± 0.0*	100.0 ± 0.0*	100.0 ± 0.0*
5	1000	100.0 ± 0.0*	100.0 ± 0.0*	100.0 ± 0.0*

* *p value < 0.05*; compared to positive control, HUVECs treated with 5 mM nicotine.

dye. Non-significant inhibitory effect of *V. cinerea* extract alone (0 to 1,000 µg/ml) for 1 and 4 days on HUVECs is shown in Figure 2. If the exposure was prolonged to 7 days, the concentration greater than 500 µg/ml showed growth reduction in a dose-dependent fashion.

Cytoprotective effect of *V. cinerea* extract

Effect of nicotine and *V. cinerea* extract to HUVEC proliferation was determined using MTT assay, which examined viable cells. However, the vacuole-like structures in endothelial cytoplasm appearing in living cells cannot be observed by MTT assay. Instead, cells were stained by crystal violet to enhance the visualization of cell component and vacuole-like features under the microscope.

In the study of the cytoprotective effect of *V. cinerea* extract from nicotine toxicity, HUVECs were incubated in the culture media containing nicotine 5 mM and various concentrations of *V. cinerea* extract. Normal endothelial cells containing no vacuole-like structure in the cytoplasm were counted and calculated as percentage comparison between untreated and treated with nicotine or nicotine and *V. cinerea* extract. Treatment of HUVECs with *V. cinerea* extract for 24, 48 and 72 h reduced vacuole-like structures that occurred in endothelial cytoplasm at various degrees in a dose- and time-dependent manner as shown in Table 2 ($p < 0.05$). With exposure time extended from 24 to 48 h, the 100, 500 and 1,000 µg/ml extract could reduce the numbers of the vacuole-like structures in the cytoplasms of HUVECs by 48.5 ± 1.0, 96.1 ± 0.4, and 100.0 ± 0.0% to 94.3 ± 0.9, 100.0 ± 0.0 and 100.0 ± 0.0%, respectively. The treatment also improved the swelling degree. Disappearance of such abnormal features from the cytoplasm of endothelial cells was observed after 72 h of treatments with all of the three concentrations of the extract. The recovery of endothelial cells treated with 5 mM nicotine and 100 µg/ml extract with no vacuole-like structures is shown in Table 2 and Figure 3.

Furthermore, the survival rate of the endothelial cells that had been exposed to 7.5 mM nicotine was significantly decreased ($p < 0.05$), while the numbers of the vacuole-like structures in their cytoplasm increased. Such changes were not reverted by extending the exposure time to the extract up to 72 h (data not shown).

Effect of *V. cinerea* extract on the expression of antioxidant gene

To determine whether the mechanism of *V. cinerea* extract occurred through a change in the antioxidant activity, mRNA of endothelial cells exposed to nicotine alone or nicotine and extract at 24, 48 and 72 h were extracted. RT-PCR was used to examine the expression of key genes regulating the intracellular antioxidant capacity, SOD, CAT, GPX and GSR normalized to the mRNA level of the housekeeping gene, β-actin. The exposure to nicotine alone or in combination with *V. cinerea* extract at 24 and 48 h show no significant difference of CAT, SOD, GPX or GSR expression compared to control, untreated cells (data not shown). The down regulation of CAT expression in the endothelial cells exposed to 5 mM nicotine alone was observed when the exposure time was extended to 72 h. No significant difference of SOD, GPX or GSR expression was found as shown in Figure 4. Interestingly, *V. cinerea* extract at the concentration of 100, 500 and 1,000 µg/ml for 72 h can draw CAT expression back to normal level. The *V. cinerea* extract might counteract the nicotine effect by modulating CAT.

Effect of *V. cinerea* extract on catalase activity

From RT-PCR results, catalase expression was recovered by the *V. cinerea* extract at the concentration of 100, 500 and 1,000 µg/ml for 72 h. The same profile of catalase activity has been found. Catalase activity was reduced in HUVECs exposed to 5 mM nicotine and *V. cinerea* extract**,** 100, 500 and 1,000 µg/ml for 72 h, was able to bring catalase activity back to 83.71 ± 4.21, 99.88 ± 4.54 and 104.40 ± 4.01%, respectively compared to the control group (Figure 5).

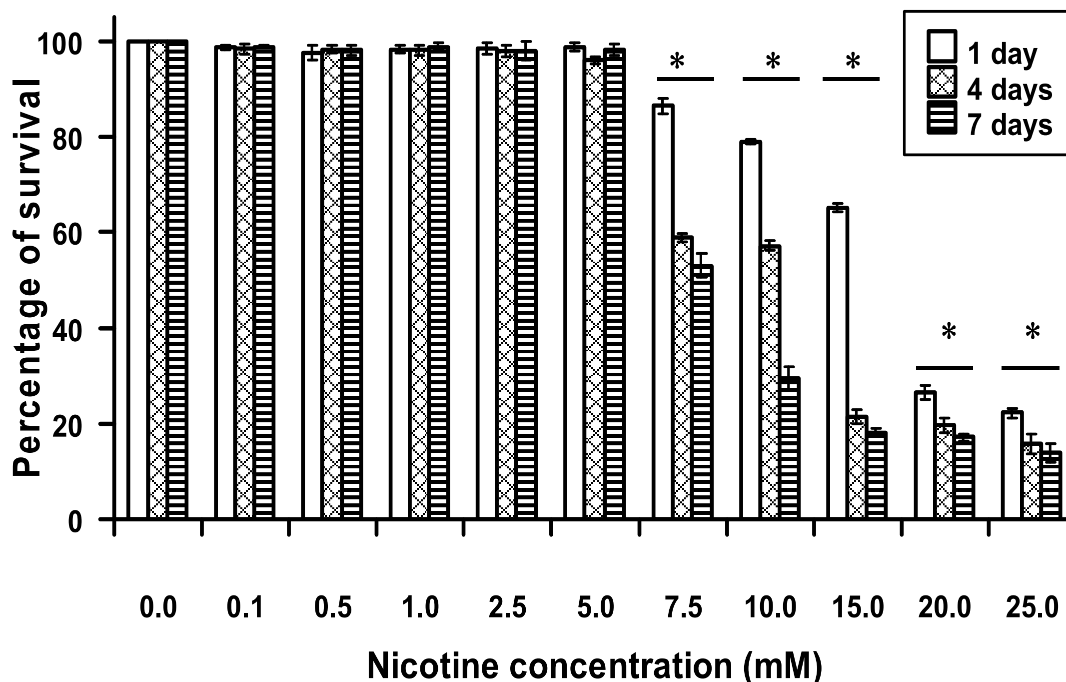

Figure 1. Effect of nicotine on proliferation of HUVECs. Endothelial cells were treated with nicotine at the concentration of 0, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, and 25.0 mM for 1, 4, and 7 days. Nicotine significantly blocked HUVEC proliferation at concentrations greater than 5 mM with both dose- and time-dependent fashion, $^{*}p < 0.05$. Percentage of survival was calculated compared to control (untreated cells). Each data point represents mean ± SD from three independent experiments. Each of the experiment was done in triplicate.

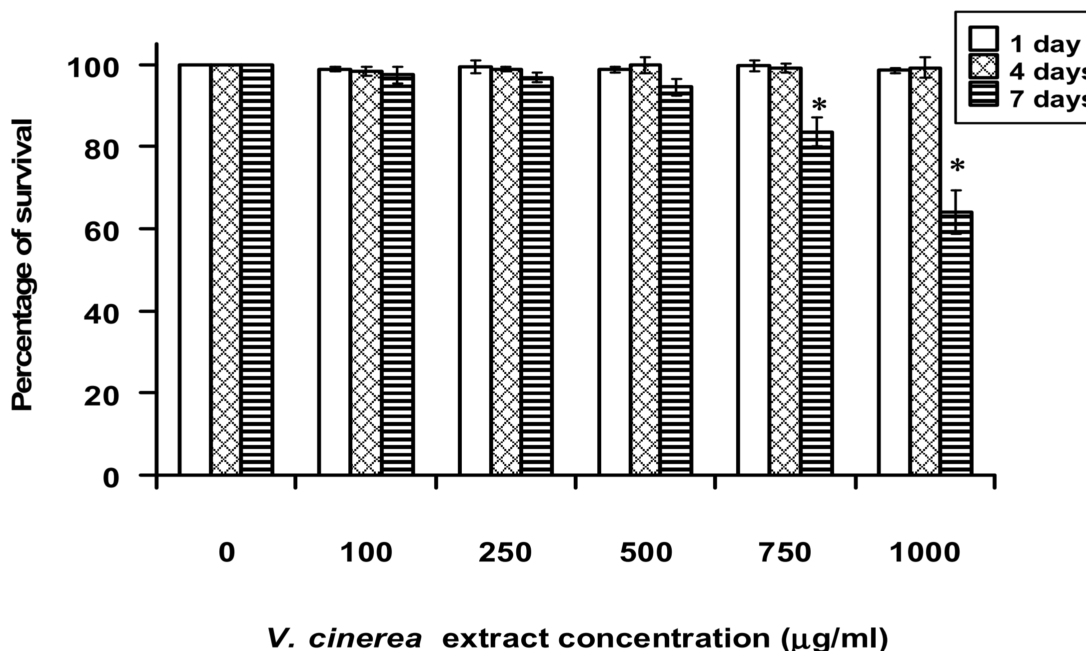

Figure 2. Effect of *V. cinerea* extract on proliferation of HUVECs. Endothelial cells were treated with *V. cinerea* extract at the concentration of 0, 100, 250, 500, 750 and 1,000 µg/ml for 1, 4 and 7 days. Growth reduction in a dose-dependent fashion was found when HUVECs was exposed to the extract for a week with the concentration greater than 500 µg/ml, $^{*}p < 0.05$. Percentage of survival was calculated compared to control (untreated cells). Each data point represents mean ± SD from three independent experiments. Each of the experiment was done in triplicate.

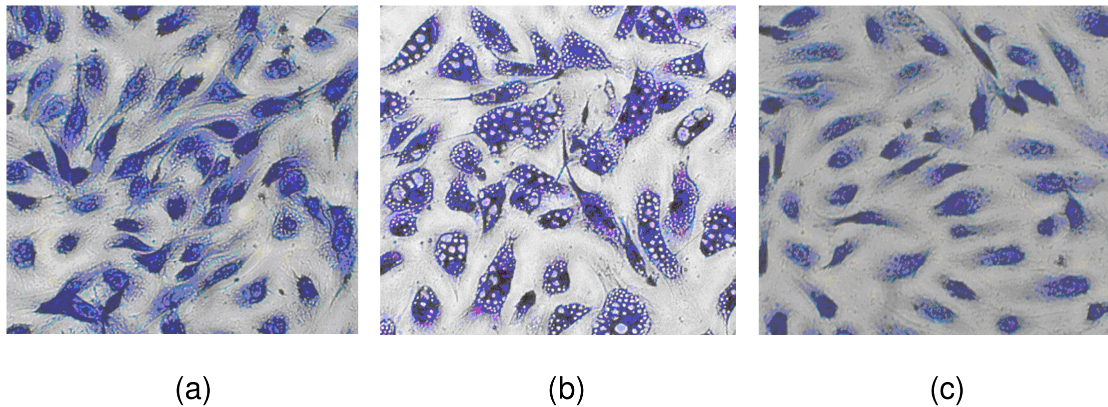

Figure 3. Cytoprotective effect of *V. cinerea* extract on vacuole-like structure of HUVECs exposed to 5 mM nicotine (40×). For (a) Control or untreated endothelial cells, (b) HUVECs treated with 5 mM nicotine for 72 h with the unusual structure in cytoplasm, vacuole-like structure, and (c) HUVECs treated with 5 mM nicotine and 100 µg/ml *V. cinerea* extract for 72 h. No vacuole-like structure was observed.

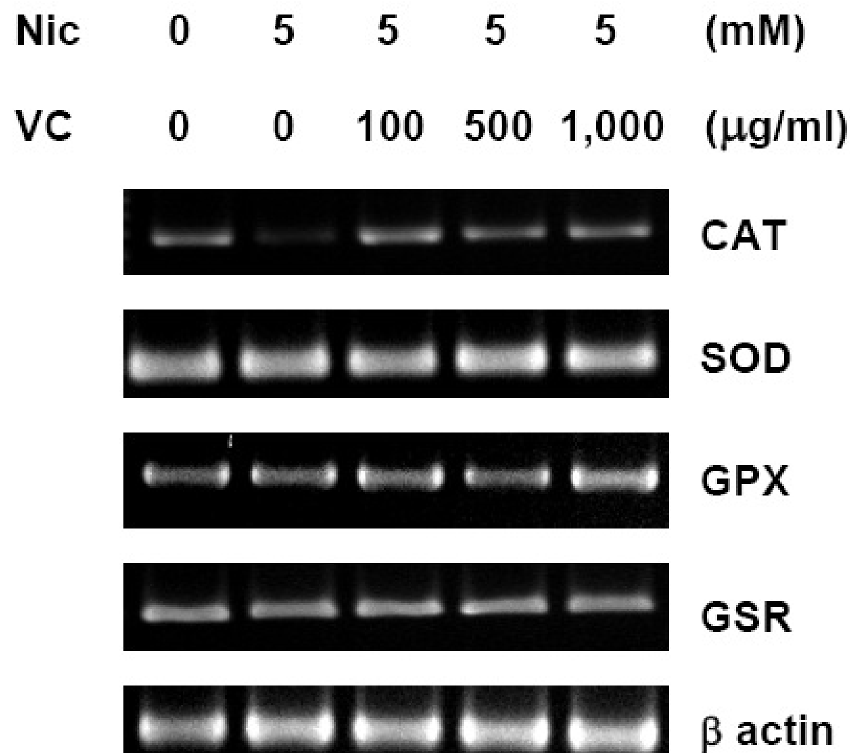

Figure 4. The effect of *V. cinerea* extract on nicotine-mediated expression of antioxidant genes in endothelial cells. HUVECs were treated with nicotine alone or in combination with *V. cinerea* extract for 72 h.

DISCUSSION

No toxic effect of 0.1 to 5 mM nicotine on HUVECs following one week exposure was observed. Increasing the nicotine concentration from 7.5 to 25 mM gradually decreased survival of HUVECs in both dose- and time-dependent manner. The abnormal features, that is, the vacuole-like structures appeared in the cytoplasms of HUVECs that had been exposed to nicotine at concentrations above 5 mM. These results indicated that the toxic effect of nicotine to HUVECs consists of a subcellular response, without cell death at 5 mM nicotine, and with different degree of cell death at the concentration higher than 5 mM.

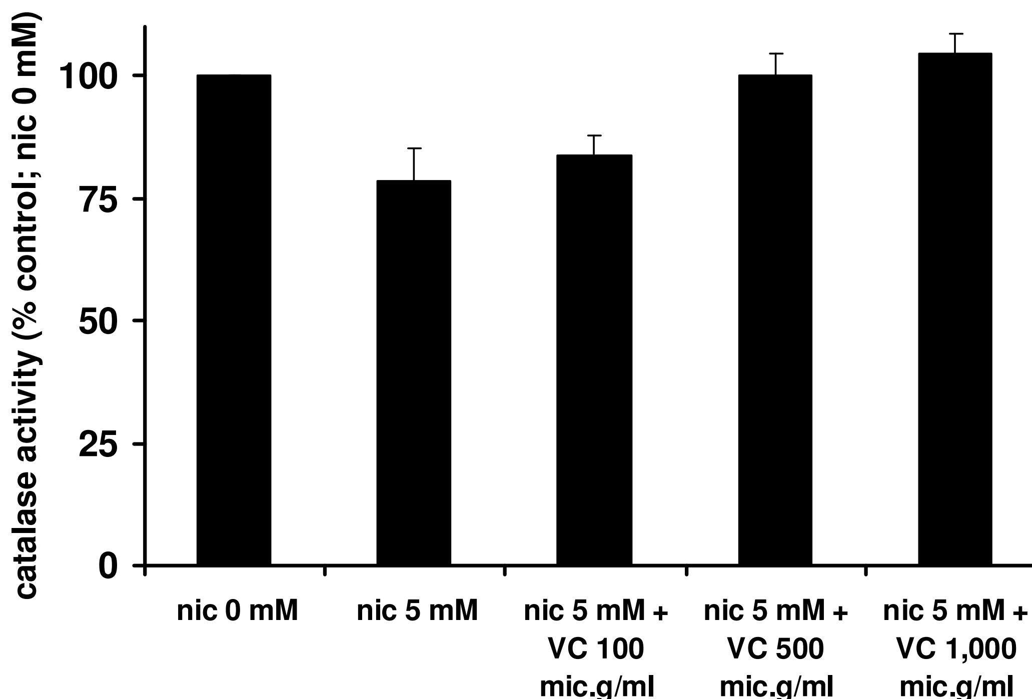

Figure 5. The effect of *V. cinerea* extract on nicotine-mediated catalase activity. HUVECs were treated with nicotine alone or in combination with *V. cinerea* extract for 72 h. Each data point represents mean ± SD from three independent experiments. Each of the experiment was done in triplicate.

Nicotine, an active component of cigarettes has been found to induce carcinogenesis, cell proliferation, invasion, and metastasis in a variety of human cancer cell lines (Dasgupta et al., 2009). Angiogenesis was confirmed to facilitate the cancer metastatic effect of nicotine. Nicotine-induced angiogenesis is achieved through the binding of nicotine to nicotinic acetylcholine receptors leading to proliferation and up-regulation of several pro- and anti-angiogenic molecules in endothelial and smooth-muscle cells contributing to tumor growth and metastasis (Jain, 2001). Nicotine was reported to induce cytoplasmic vacuolization in endothelial cells (Park et al., 2008).

Vascular endothelial growth factor (VEGF) can induce a lumen-like structure formed by several endothelial cells such as bovine skeletal muscle endothelial cells (BSMEs). The coalescence of vacuoles within a single BSME leads to tube-like structures in tube formation process of angiogenesis (Yang et al., 2001). Under angiogenic conditions, the majority of invading endothelial cells having vacuole-like structures rapidly differentiates forming tube-like structures with multi-cellular walls turning into a new vessel. The mechanism of vacuolar formation in endothelial cytoplasm remains unclear. The coalescence of vacuoles in endothelial cell might be essential for tube-like structures prior to tube formation (Kamei et al., 2006). The inhibition of vacuolar formation in endothelial cells could therefore constitute a strategy for arresting angiogenesis.

V. cinerea extract alone at the concentrations between 100 and 1,000 µg/ml did not affect the proliferations of HUVECs after 1 and 4 days of treatment. The obvious growth reduction was found when the concentration was higher than 500 µg/ml for a week. The concentration of *V. cinerea* extract in serum or after making it bioavailable via drinking or other route of administration should be much lower than 100 µg/ml. As for the safety of *V. cinerea* extract to the endothelial cells, no significant toxicity has been reported in brine shrimp or mice (Latha et al., 2010).

The observations in this study showed significant alteration of the subcellular response in HUVEC cytoplasm against nicotine. Intracellular vacuole-like structures in HUVECs exposed to mixture of 5 mM nicotine and various concentrations of *V. cinerea* extract were decreased in a dose-dependent fashion and completely disappeared if the exposed time was extended to 72 h with low concentration of extract of 100 µg/ml. Vacuole-like structures also disappeared with shorter exposure time of 24 or 48 h, but concentration of the extract higher than 100 µg/ml were required. These data implies a cytoprotective potential of *V. cinerea* extract against nicotine at 5 mM. This originates a new concept of using *V. cinerea* extract for arresting vacuolar formation, which is the hallmark of tube-like structures in angiogenic process. *In vitro* angiogenic assay merits further studies to demonstrate the applicability of this extract for antiangiogenic property.

The antioxidative activity of *V. cinerea* was found to scavenge both hydroxyl and superoxide radicals, as well as significantly inhibit lipid peroxidation possibly via endogenous antioxidant enzymes such as catalase, superoxide dismutase, glutathione, glutathione peroxidase and glutathione-S transferase (Pratheeshkumar and Kuttan, 2009). The results from RT-PCR indicated that the capacity of this extract to reduce vacuole-like structure in HUVECs may be partly due to the modulation of a key gene regulating intracellular antioxidant capacity: catalase gene and catalase activity. Hydrogen peroxide might be involved in the formation of vacuole-like structure. It is, therefore, possible that the *V. cinerea* extract contains intracellular antioxidant activities in endothelial cell.

With higher concentration of 7.5 mM nicotine, cytoplasmic vacuoles were markedly increased and unable to be removed by high concentration of *V. cinerea* extract, 1,000 µg/ml (data not shown). In this condition, the degree of damage occurring via cytoplasmic vacuole may be irreversible.

Conclusion

The results of this research showed that the *V. cinerea* extract may be safe for the HUVECs. Furthermore, the abnormal vacuole-like structures in their cytoplasms of cells exposed to 5 mM nicotine could be significantly attenuated with 100 to 1,000 µg/ml of the extract in a dose- and time-dependent manner. The ability of *V. cinerea* extract to inhibit vacuolar-like structure formation was probably due to the modulation of catalase attributable to tube formation and angiogenic blockade. Angiogenic assay, endothelial migration, invasion and tube formation assay, should be extensively explored *in vivo*.

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