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Effect of *Fimbristylis ovata* on receptor for advanced glycation end-products, proinflammatory cytokines, and cell adhesion molecule level and gene expression in U937 and bEnd.3 cell lines

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ABSTRACT. *Fimbristylis ovata* has been long used as a traditional medicine for chronic inflammatory diseases; however, there are no data regarding its anti-inflammatory properties. In this study, we investigated the effects of *F. ovata* extracts on the secretion of pro-inflammatory cytokines, cell adhesion molecule, and receptor for advanced glycation end-products (RAGE) in lipopolysaccharide-stimulated cells. *F. ovata* was extracted using the maceration method with 3 different solvents: ethanol, methanol, and water. The effect of *F. ovata* extracts on cell viability was evaluated using the MTT assay. Pro-inflammatory cytokines and cell adhesion molecules were investigated by reverse transcription-polymerase chain reaction and an enzyme-linked immunosorbent assay. Upon incubation with *F. ovata* extracts up to 100 μg/mL, cell viability was more than 80%. *F. ovata* extracts could inhibit interleukin-6 level and gene expression as well as the RAGE gene in the

monocytic cell lineU937. Moreover, the results showed that vascular cell adhesion molecule 1 secretion and gene expression were decreased when lipopolysaccharide-activated brain endothelial cells (bEnd.3) were treated with *F. ovata* extracts. Therefore, the anti-inflammatory activity of *F. ovata* extracts may result from their inhibitory actions via the RAGE signaling pathway.

Key words: bEnd.3; Cell adhesion molecule; *Fimbristylis ovata*; U937; Proinflammatory cytokines; Random activation of gene expression

INTRODUCTION

Chronic inflammation is a major cause of chronic degenerative diseases, including rheumatoid arthritis, systemic lupus erythematosus, cardiovascular disease, cancer, and type 2 diabetes mellitus. Several studies have demonstrated that excessive production of pro-inflammatory cytokines and chemokines are involved in the pathogenesis of these diseases (Sweeney and Firestein, 2004; Davis et al., 2008; King, 2008; Van den Oever et al., 2010). Furthermore, upregulation of adhesion molecules in endothelial cells during leukocyte recruitments associated with the progression of atherosclerosis and systemic lupus erythematosus (Ballantyne and Nambi, 2005; Rhew and Ramsey-Goldman, 2006). Endothelial dysfunction plays a role in the development of diabetic complications (Esper et al., 2008). Several studies have found that activation of receptor for advanced glycation end-products (RAGE) contributes to inflammation, development of diabetic complications, and cardiovascular disease (Lukic et al., 2008; Yan et al., 2009). Activation of RAGE has been demonstrated to mediate various intracellular pathways, including phosphoinositide 3-kinase/AKT, mitogen-activated protein kinase, and nuclear factor (NF)-κB, which lead to the secretion of pro-inflammatory cytokines (Lin et al., 2009).

An increasing number of studies have reported novel therapeutic interventions for chronic inflammatory diseases. Accumulating evidence revealed that anti-interleukin-6 (IL-6) therapies and blockade of the IL-6 receptor using a neutralizing monoclonal antibody may suppress chronic inflammation in degenerative diseases, such as some types of cancers (Tawara et al., 2011), rheumatoid arthritis, Castleman's disease, and juvenile idiopathic arthritis (Neurath and Finotto, 2011). Another study indicated that inhibition of tumor necrosis factor-alpha (TNF-α) receptor and chimeric monoclonal antibody TNF-α can be used to treat diabetic complications (Navarro-González et al., 2009). Moreover, TNF-α inhibitors are available for treating rheumatoid arthritis (Thalayasingam and Isaacs, 2011). It was previously shown that blocking of IL-1β activity could reduce the severity of acute and chronic auto-inflammatory diseases. The IL-1 receptor antagonist was approved for treatment of rheumatoid arthritis and type 2 diabetes mellitus (Dinarello, 2011). The soluble extracellular domain of RAGE can be used as a potential therapeutic blocker in the treatment of diabetic macro vascular diseases and cardiovascular diseases (Park et al., 1998). We also found that alternately spliced RAGEv1 or soluble RAGE can decrease the expression of NF-κB and TNF-α in HepG2 cells, suggesting that it also has a therapeutic effect in hepatic cancer (Lertwittayapon et al., 2012). Recent studies have shown that glucagon-like peptide-1, a therapeutic target for the treatment of patients with type 2 diabetes, may downregulate vascular cell adhesion molecule 1 (*VCAM-1*) mRNA levels by reducing RAGE gene expression (Ishibashi et al., 2010). Traditional Chinese medicine used for treating inflammatory processes and atherosclerosis may reduce *VCAM-1*

gene expression (Kwon et al., 2007). Many Thai herbs have been used for therapeutic purposes in traditional medicine. Several studies have demonstrated that *Boesenbergia rotunda* (L.) Mansf. has an anti-inflammatory effect, which may be derived from its active flavonoid derivatives (Tuchinda et al., 2002; Ching et al., 2007). *Lagerstroemia speciosa* (L.) and *Piper sarmentosum* Roxb. exert pharmacological effects such as anti-inflammatory effects and antioxidant activity (Priya et al., 2008; Hussain et al., 2009).

Fimbristylis ovata (Burm.f.) Kern., known by its common name flat spike sedge or "Ya-sae-ma" in Thai, belongs to the family Cyperaceae (Simpson and Koyama, 1998). Plants in the family Cyperaceae consist of several components such as phenolic compounds, flavonoids, alkaloid, glycoside proteins, amino acids, tannins, and saponins (Chaulya et al., 2010, 2011). Our previous study demonstrated that *F. ovata* has antioxidant activity and inhibits RAGE gene expression in a human lung adenocarcinoma epithelial cell line (Sukjamnong and Santiyanont, 2012). Moreover, *F. ovata* has been long used for treating adenitis, scrofula, syphilis, cough, bronchitis, and asthma (Burkill et al., 1985; Khare, 2007), but the underlying mechanism of its therapeutic activity is unknown. The aim of this study was to investigate the effects of *F. ovata* extracts on the secretion of pro-inflammatory cytokines, cell adhesion molecules, and activation of RAGE in lipopolysaccharide (LPS)-stimulated cells.

MATERIAL AND METHODS

Plant material

F. ovata was collected from a single source in Bangkok, Thailand, and identified by Professor Kasin Suvatabhandhu of the Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand, voucher No. 013431(BCU). The fresh plant (stem and flower) was cleaned with water, cut into short pieces, dried in a laboratory oven at 45°C for 5 days, and finally ground into a fine powder. The plant powder was extracted with 1:10 (v/v) ethanol or 1:10 (v/v) methanol by maceration in a shaking incubator at 37°C for 72 h. The extract was filtered and processing was repeated twice, and all filtered extracts were collected and evaporated using a rotatory evaporator. The plant powder was also extracted with 1:10 (v/v) water by boiling at 100°C for 2 h followed by lyophilization. Crude extracts were dissolved in dimethyl sulfoxide and maintained as 100 mg/mL stock solutions at -20°C and protected from light.

Cell culture

Human monocytic cells (U937) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycinin in a humidified atmosphere with 5% CO_2 at 37°C. Mouse brain endothelial cells (bEnd.3) were cultured under the same conditions except that the medium used was Dulbecco's modified Eagle medium (DMEM-high glucose, Gibco, Grand Island, NY, USA).

MTT assay for cell viability

Cell viability was determined using the MTT assay (Wang et al., 2011) to measure mi-

tochondrial dehydrogenase enzyme activity that reduces 3-(4,5-dimethyl-triazolyl-2-yl)-2,5-diphenyl tetrazolium bromide to purple formazan. Cells were seeded on 96-well plates at a density of 5 x 10^3 cells/well and incubated in a humidified 5% CO_2 incubator at 37°C for 24 h. Cells without *F. ovata* extracts were used as a negative control and considered as 100% viable. *F. ovata* extracts were added and incubated for 24 h. Next, 20 µL 0.5 mg/mL MTT was added and the plates were incubated for 4 h in a humidified 5% CO_2 at 37°C. The culture medium was removed and 200 µL dimethyl sulfoxide was added to dissolve the formazan crystals. Absorbance was measured at 550 nm using a microplate reader. The percentage of cell viability was calculated based on the following formula:

% cell viability = [(absorbance of treatment group - blank) / (absorbance of control group - blank)] x 100.

Enzyme-linked immunosorbent assay (ELISA)

U937 cells were seeded at a density of 5 x 10^5 cells/mL on 24-well plates and 10 ng/mL phorbol 12-myristate 13-acetate was added to activate the differentiation of cells into macrophages (Grkovich et al., 2006). *F. ovata* extracts were added and the cells were incubated for 6 h, then 1 µg/mL LPS was added and the cells were incubated for 24 h. The supernatants were collected and stored at -20°C. The cultured supernatants were measured for human TNF-α, human IL-1β, and human IL-6 using an ELISA kit (R&D Diagnostics, Minneapolis, MN, USA) according to the manufacturer protocol.

bEnd.3 cells were seeded at a density of 5 x 10^5 cells/mL on 24-well plates. *F. ovata* extracts were added and the cells were incubated for 6 h. Next, 1 µg/mL LPS was added and the cells were incubated for 24 h. The supernatants were collected and stored at -20°C. The cultured supernatants were measured for mouse VCAM-1 using the ELISA kit as described above.

Reverse-transcription polymerase chain reaction analysis

U937 cells were seeded in density of 1 x 10^6 cells/mL in 6-well plates and 10 ng/mL phorbol 12-myristate 13-acetate was added to differentiate cells into macrophages. bEnd.3 cells were seeded at a density of 1 x 10^6 cells/mL in 6-well plates. *F. ovate* was added and the cells were incubated for 6 h, then 1 µg/mL LPS was added and the cells were incubated for 24 h. Total RNA was extracted from the cells using Tri-RNA reagent (Favorgen Biotech Corp., Taiwan, China) according to manufacturer instructions. RNA was treated with deoxyribonuclease I (DNase I; Promega, Madison, WI, USA). DNase I-treated RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega) following the manufacturer protocol. For the amplification reaction, a polymerase chain reaction mixture composed of 5 µL reverse transcription reaction mixture, 10X Taq buffer, 1.0 mM $MgCl_2$, 10 mM dNTPs, 10 µM primers, and 1.25 U *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania) was used. To amplify cDNA, each desired DNA fragment was amplified for 35 cycles using each gene-specific primer pair listed in Table 1 (Jang et al., 2006; Park et al., 2009). The product was separated on a 2% agarose gel, stained with ethidium bromide, and visualized using a gel documentation system (SynGene, UK).

Table 1. List of gene-specific primers for reverse transcription-polymerase chain reaction.

Gene		Sequence	Annealing temp (°C)	Product size (bp)
β-actin	Forward primer	5' ACGGGTCACCACACTGTGC 3'	58	656
	Reverse primer	5' CTAGAAGCATTTGCGGTGGACGATG 3'		
IL-1β	Forward primer	5' AGCCATGGCAGAAGTACCT 3'	50	823
	Reverse primer	5' CAGCTCTCTTTAGGAAGACAC 3'		
IL-6	Forward primer	5' ATGAACTCCTTCTCCACAAGCGC 3'	50	252
	Reverse primer	5' GAAGAGCCCTCAGGCTGGACTG 3'		
TNF-α	Forward primer	5' TCTCGAACCCCGAGTGACAA 3'	55	181
	Reverse primer	5' TGAAGAGGACCTGGGAGTAG 3'		
VCAM	Forward primer	5' AGGCACAGCTGCAGGATGCC 3'	59	333
	Reverse primer	5' GGAGGGGGCGGGGCTGTAAT 3'		
RAGE	Forward primer	5' GTGGGGACATGTGTGTCAGAGGGAA 3'	65	383
	Reverse primer	5' TGAGGAGAGGGCTGGGCAGGGACT 3'		

Statistical analysis

Data are reporetd as means ± standard error of the mean of 3 experiments. Statistically significant differences were determined by one-way analysis of variance followed by a *post-hoc* Tukey test. Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

Effect of *F. ovata* extracts on cell viability in U937 and bEnd.3 cells

Based on the results of the MTT assay, upon incubation of U937 and bEnd.3 cells with various concentrations of *F. ovata* extracts for 24 h, the extracts showed no significant effect on cell viability of U937 and bEnd.3 in the concentration range 0.19-100 μg/mL. Cell viability was greater than 80% (Figure 1).

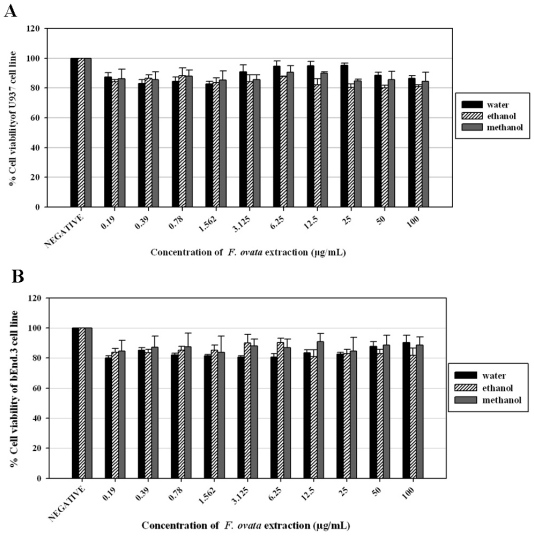

Figure 1. Cell viability was determined using the MTT assay. Cells were incubated with *F. ovata* extracted by water, ethanol, and methanol. **A.** U937; **B.** bEnd.3. Negative included cells without *F. ovata* extracts. Data are reported as means ± standard error of the mean of triplicate measurements.

Effects of *F. ovata* extracts on proinflammatory cytokines and adhesion molecule levels

Proinflammatory cytokines and adhesion molecule levels were measured using ELISA. After incubating the cells with various concentrations of *F. ovata* extracts for 24 h, we found that IL-6 and VCAM-1 concentrations were significantly decreased ($P \leq 0.05$) compared with the control cells that had not been exposed to the extracts, as shown in Figures 2 and 3.

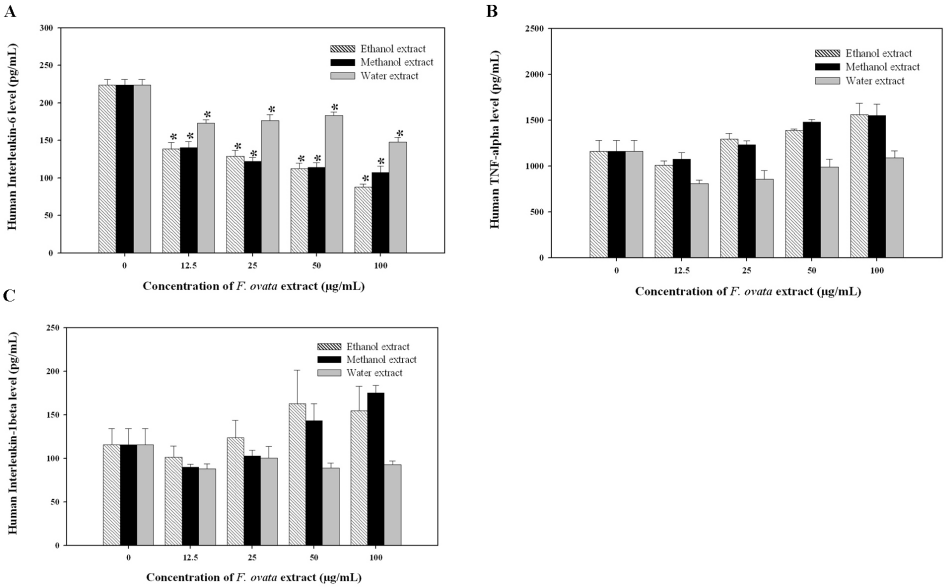

Figure 2. Effect of *Fimbristylis ovata* extracts on IL-6 (**A**), TNF-α (**B**), and IL-1β (**C**) secretion in LPS-stimulated U937 cells compared for extract-treated cells and non-treated control cells. Data are reported as means ± standard error of the mean for triplicate measurements ($*P \leq 0.05$).

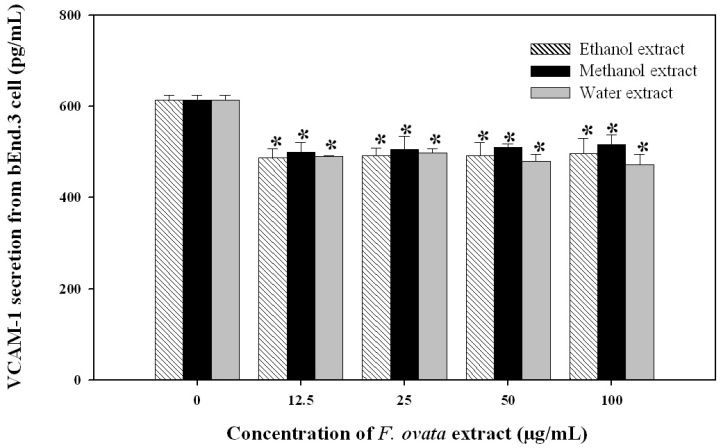

Figure 3. Effect of *Fimbristylis ovata* extracts on VCAM-1 secretion in LPS-stimulated bEnd.3 cells compared for extract-treated cells and non-treated control cells. Data are reported as means ± standard error of the mean for triplicate measurements ($*P \leq 0.05$).

F. ovata extracts downregulate gene expression of *IL-6*, *RAGE*, and *VCAM-1*, but not of *TNF-α* and *IL-1β*

Gene expression was determined by reverse-transcription polymerase chain reaction. After incubating U937 cells with various concentrations of *F. ovata* extracts for 24 h, *IL-6* and *RAGE* gene expression were significantly decreased ($P \leq 0.05$) compared with the control cells. However, *TNF-α* and *IL-1β* mRNA expression did not decrease, as shown in Figures 4 and 5. Regarding the effect of *F. ovata* extracts on cell adhesion molecule in endothelial cells, after bEnd.3 cells were incubated with *F. ovata* ethanol and methanol extracts for 24 h, *VCAM-1* gene expression was significantly decreased ($P \leq 0.05$) compared with the control cells (Figure 6).

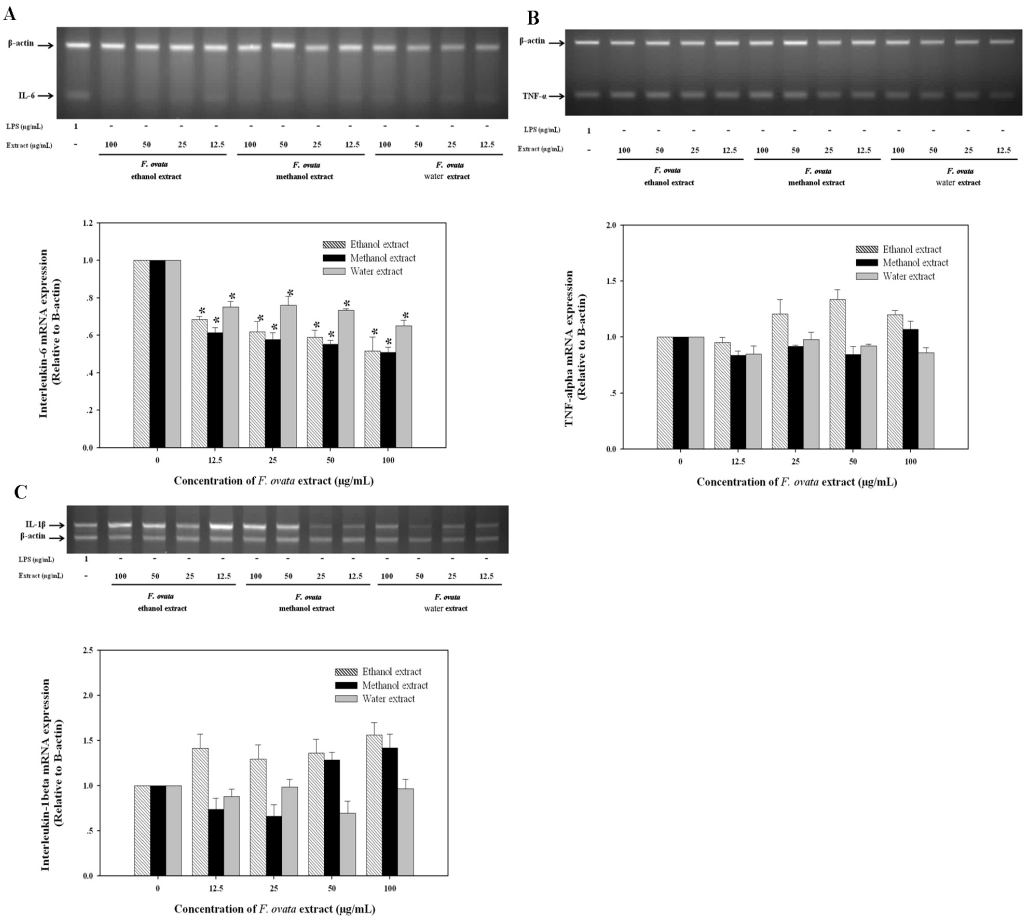

Figure 4. Effect of *Fimbristylis ovata* extracts on *IL-6*, *TNF-α*, and *IL-1β* gene expression in LPS-stimulated U937 cells compared for extract-treated cells and non-treated cells. **A.** *IL-6*; **B.** *TNF-α*; **C.** *IL-1β*. *β-actin* was used for normalization. Data are reported as means ± standard error of the mean of triple measurements (*$P \leq 0.05$).

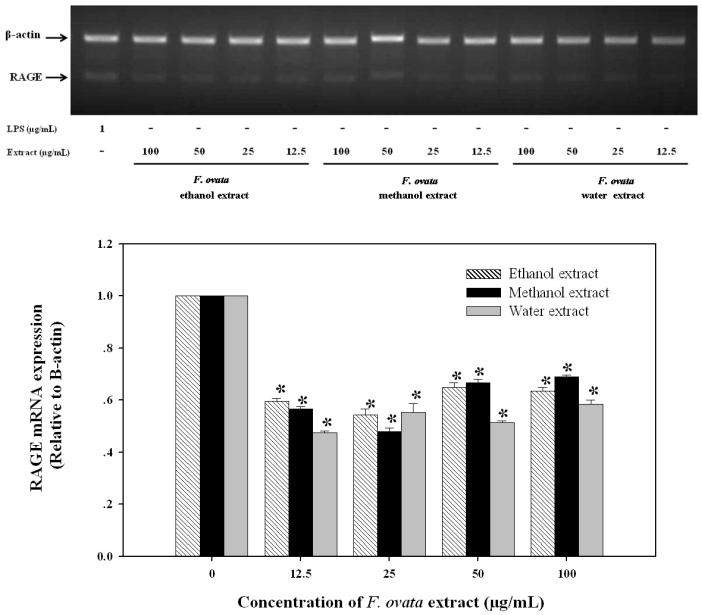

Figure 5. Effect of *Fimbristylis ovata* extracts on *RAGE* gene expression in LPS-stimulated U937 cells compared for extract-treated cells and non-treated cells. *β-actin* was used for normalization. Data are reported as means ± standard error of the mean of triplicate measurements (*P ≤ 0.05).

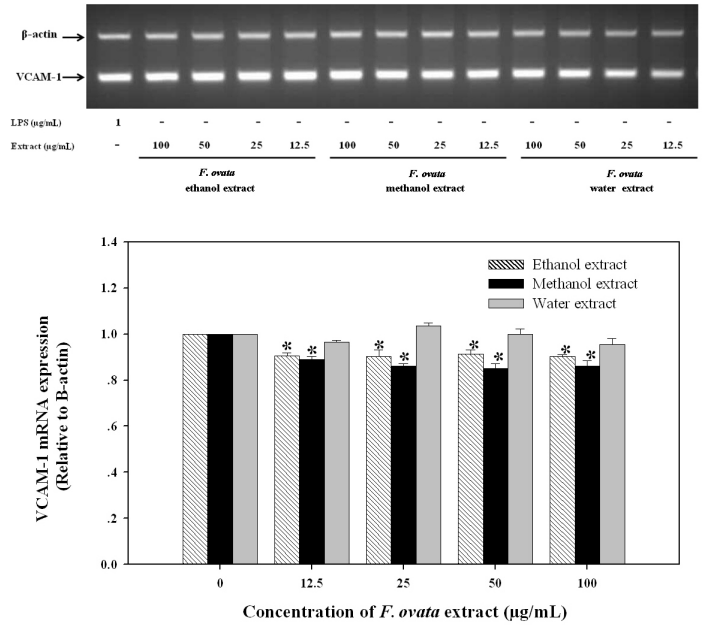

Figure 6. Effect of *Fimbristylis ovata* extracts on *VCAM-1* gene expression in LPS-stimulated bEnd.3 cells compared for extract-treated cells and non-treated cells. *β-actin* was used for normalization. Data are reported as means ± standard error of the mean for triplicate measurements (*P ≤ 0.05).

DISCUSSION

This is the first report of the possible anti-inflammatory mechanism and therapeutic effect of *F. ovata*. There was no difference in cell viability after treating cells with various extracts of *F. ovata*. Production of the pro-inflammatory cytokine IL-6 in U937 cells was significantly decreased in a dose-dependent manner with *F. ovata* extracts using water, ethanol, and methanol. At concentrations of 12.5-100 μg/mL, the extracts of all solvents inhibited *IL-6* mRNA expression in a dose-dependent manner, but did not reduce TNF-α or IL-1β secretion and mRNA expression. In addition, production of the cell adhesion molecule VCAM-1 in bEnd.3 cells was significantly decreased by the extracts at concentrations of 12.5-100 μg/mL, which has been demonstrated for both secretion and mRNA expression. Importantly, these concentrations of *F. ovata* extracts also inhibited *RAGE* mRNA expression. Activation of RAGE transduced cell surface signals involved in various intracellular pathways, including phosphoinositide 3-kinase/AKT, mitogen-activated protein kinase, and NF-κB (Lin et al., 2009). Additional studies demonstrated that the expression of IL-6 was induced through RAGE-activated pathways, including NF-κB and mitogen-activated protein kinase signaling pathways (Rasheed et al., 2011). Moreover, activation of RAGE has been shown to induce expression of VCAM-1 by the NF-κB signaling pathway (Schmidt et al., 1995). Therefore, *F. ovata* extracts may play a protective role against inflammation by suppressing gene expression and producing pro-inflammatory cytokines as well as cell adhesion molecules by downregulating *RAGE* gene expression (Kwon et al., 2007; Ishibashi et al., 2010). Kimura et al. (2005) reported that LPS directly activated Jak2 and Stat5, resulting in specific recruitment of Stat5 to the *IL-6* promoter together with the NF-κB p50 subunit. Evidence suggests that IκBζ associated with the NF-κB p50 subunit activates the promoter region of *IL-6* (Lu et al., 2008). In addition, transcription factor CCAAT enhancer binding protein β has been shown to be critically involved in regulating IL-6 production (Bretz et al., 1994). Based on this study, the effect of *F. ovata* extracts on the secretion of IL-6 may be related to its inhibitory actions via the RAGE signaling pathway, Jak2-Stat5 pathway, IκBζ, and CCAAT enhancer binding protein β. Additional studies are necessary to elucidate the effect of *F. ovata* extracts on the regulation of these signaling pathways. In conclusion, our results showed that *F. ovate* not only suppressed cytokine and cell adhesion molecule secretion, but also inhibited RAGE expression. This suggests that *F. ovata* may be useful for treating chronic inflammation. However, the effect of *F. ovata* on downstream signaling of the RAGE signaling pathway should be further examined.

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