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Antithrombotic and Profibrinolytic Activities of Eckol and Dieckol

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ABSTRACT
In order to develop new anticoagulant agents, two single compounds (eckol and dieckol) were isolated from *Eisenia bicyclis* and examined their anticoagulant activities by monitoring activated partial thromboplastin time (aPTT), prothrombin time (PT) as well as cell-based thrombin and activated factor X (FXa) generation activities. And the effects of eckol and dieckol on the expression of plasminogen activator inhibitor type 1 (PAI-1) and tissue-type plasminogen activator (t-PA) were tested in tumor necrosis factor-α (TNF-α) activated human umbilical vein endothelial cells (HUVECs). Data showed that eckol and dieckol prolonged aPTT and PT significantly and inhibited thrombin and FXa activities. They also inhibited the generation of thrombin or FXa in HUVECs. In accordance with these anticoagulant activities, eckol or dieckol showed anticoagulant effect in vivo. Furthermore, eckol and dieckol inhibited TNF-α induced PAI-1 production and the ratio between PAI-1 and t-PA was found to be significantly decreased by eckol and dieckol. Surprisingly, these anticoagulant and profibrinolytic effects of dieckol were better than those of eckol indicating that hydroxyl group in eckol positively regulated anticoagulant function of eckol. Therefore, these results suggest that eckol or dieckol possesses antithrombotic activities and provides a possibility to develop as an agent for the anticoagulation. J. Cell. Biochem. 113: 2877–2883, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: ECKOL; DIECKOL; COAGULATION CASCADE; FIBRINOLYSIS; ENDOTHELIUM

The vascular endothelium plays a pivotal role in all major pathways involved in the pathogenesis of the hemostatic derangement observed during severe inflammation (i.e., initiation and regulation of thrombin generation and fibrinolysis) [Esmon, 2001]. Damage to the endothelium exposes a procoagulant factor known as "tissue factor (TF)". TF binds to activated factor VII and the resulting complex activates factor X (FXa). Then, FXa converts prothrombin into thrombin, which cleaves fibrinogen into fibrin, a blood clot. At the same time, the fibrinolytic system is inhibited. Cytokines and thrombin stimulate the release of plasminogen-activator inhibitor-1 (PAI-1), from platelets and the endothelium [Esmon, 2001; Furie and Furie, 2005]. When a clot forms, it is ultimately broken down plasmin, which is activated by tissue-type plasminogen activator (t-PA) from plasminogen. Thrombin, itself, is an activator of inflammation and inhibitor of fibrinolysis [Esmon, 2001; Furie and Furie, 2005].

Thrombin is an important enzyme for all major thrombotic processes, including physiologic hemostasis and pathologic thrombosis and this enzyme is the production of thrombin, which is required for the conversion of fibrinogen to fibrin [Davie, 1995]. Thrombin resides in the cell in an inactive form, called prothrombin, and is activated by the coagulation cascadevia formation of a

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complex called the prothrombin activator complex [Davie et al., 1991; Davie, 1995; Monroe et al., 2002]. The formation of the prothrombin activator complex occurs by two different pathways: the intrinsic prothrombin activation pathway and the extrinsic prothrombin activation pathway. Though the ultimate goal of both the pathways is the generation of the prothrombin activator complex, alternate routes are used, each giving rise to a different form of the prothrombin activator [Davie et al., 1991; Davie, 1995; Monroe et al., 2002]. The clotting time assay measures the lag time of thrombin generation and the activated partial thromboplastin time (aPTT) is a performance indicator measuring the efficacy of both the contact activation pathway and the common coagulation pathways [Quinn et al., 2000]. Further, the prothrombin time (PT) is measure of the extrinsic pathway of coagulation [Della Valle et al., 1999; Furie and Furie, 2005].

Marine natural products provide a rich source of chemical diversity and are a rich some biologically active materials that can be used to design and develop new, potentially useful therapeutic agents [Li et al., 2009]. In this study, therefore, we tried to screen active compounds from *Eisenia bicyclis* and tested their anticoagulant activities and such as the generation of FXa and thrombin as well as the regulation of clotting time (PT and aPTT) and fibrinolytic activities.

MATERIALS AND METHODS

REAGENTS

TNF-α was purchased from R&D Systems (Minneapolis, MN). Anti-TF antibody was purchased from Santa Cruz Biologics (Santa Cruz, CA). Factor V, Vll, Vlla, FX, FXa, antithrombin III (AT III), prothrombin and thrombin were obtained from Haematologic Technologies (Essex Junction, VT). aPTT assay reagent and PT reagents were purchased from Fisher Diagnostics (Middletown, VA). Chromogenic substrates S-2222, and S-2238 were from Chromogenix AB (Sweden). The PAI-1 and t-PA ELISA kits were from American Diagnostica Inc (Stamford, CT). Other reagents were special grade as commercially available.

PLANT MATERIALS, EXTRACTION AND ISOLATION

Fresh *E. bicyclis* was washed three times with water to remove salt. Lyophilized *E. bicyclis* was ground into powder before extraction. The dried *E. bicyclis* powder (1.0 kg) was extracted with MeOH (10 L × 3) at room temperature and the solvent was evaporated in vacuo. The combined crude MeOH extract (164.3 g) was suspended in 10% MeOH (1.0 L), and then partitioned in turn with *n*-hexane (1.0 L × 3), CH_2Cl_2 (1.0 L × 3), EtOAc (1.0 L × 3), and *n*-BuOH (1.0 L × 3) to yield dried *n*-hexane- (42.3 g), CH_2Cl_2 (2.5 g), EtOAc- (23.0 g), *n*-BuOH (26.5 g) and H_2O-soluble (69.1 g) residues. A portion (10.0 g) of the EtOAc extract was chromatographed on a Sephadex LH-20 column (4.0 cm i.d. × 50 cm) with MeOH and fractioned into seven subfractions (EB01 – EB07). Subfractions EB02 and EB07 were subjected to column chromatography over a LiChroprep RP-18 column (1.1 cm i.d. × 37 cm) with aqueous MeOH to yield pure eckol (1) (t_R 4.0 min, 25.2 mg) and dieckol (2) (t_R 8.1 min, 17.2 mg).

ANTICOAGULATION ASSAY

Determination of aPTT and PT were performed according to the manufacture's specifications using Thrombotimer (Behnk Elektronik, Germany). In brief, citrated normal human plasma (90 μl) was mixed with 10 μl of eckol or dieckol and incubated for 1 min at 37°C. Then, aPTT assay reagent (100 μl) was added to the mixture and incubated for 1 min at 37°C. Thereafter, 20 mM $CaCl_2$ (100 μl) was added and the clotting time was recorded. For the PT assay, citrated normal human plasma (90 μl) was mixed with 10 μl of a eckol or dieckol stock and incubated for 1 min at 37°C. Then, PT assay reagent (200 μl), preincubated for 10 min at 37°C, was added and the clotting time was recorded. PT results expressed in seconds and INR (International Normalized Ratio) and aPTT results expressed in seconds. $INR = (PT\ sample/PT\ control)^{ISI}$. ISI = international sensitivity index.

CELL CULTURE

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained as described before [Bae and Rezaie, 2008]. Briefly, cells were cultured to confluency at 37°C at 5% CO_2 in EBM-2 basal media supplemented with growth supplements (Cambrex Bio Science).

THROMBIN-CATALYZED FIBRIN POLYMERIZATION

Thrombin-catalyzed polymerization was monitored every 6 s for 20 min as the change of turbidity at 360 nm in spectrophotometer (TECAN, Switzerland) at ambient temperature. Control plasma and plasma incubated with eckol or dieckol were trebly diluted TBS (50 mM Tris-buffered physiological saline solution pH 7.4) and clotted with thrombin (final concentration – 0.5 U/ml). The maximal velocity of polymerization (V_{max}, Δ_mOD/min) was recorded for each absorbance curve [Nowak et al., 2007]. All experiments were performed three times.

FACTOR Xa GENERATION ON THE SURFACE OF HUVECs

HUVECs were preincubated with indicated concentrations of eckol or dieckol for 10 min. TNF-α (10 ng/ml for 6 h in serum-free medium) stimulated confluent monolayers of HUVECs in a 96-well culture plate were incubated with FVIIa (10 nM) in buffer B for 5 min at 37°C in presence or absence of anti-TF IgG (25 μg/ml). FX (175 nM) was then added to the cells (final reaction mixture volume, 100 μl) and incubated for 15 min. The reaction was stopped by adding buffer A containing 10 mM EDTA and the amount of FXa generated in the reaction period was measured by using a chromogenic substrate, and the change in absorbance at 405 nm was monitored in a microplate reader for 2 min. The initial rate of color development was converted into FXa concentrations from a standard curve prepared with known dilutions of purified human FXa.

THROMBIN GENERATION ON THE SURFACE OF HUVECs

HUVECs were preincubated in 300 μl containing eckol or dieckol in 50 mM Tris–HCl buffer, 100 pM FVa and 1 nM FXa for 10 min and prothrombin was added to a final concentration of 1 μM. After 10 min, duplicate samples (10 μl each) were transferred to a 96-well plate containing 40 μl of 0.5 M EDTA in Tris-buffered saline in each

well to terminate prothrombin activation. Activated prothrombin was determined by measuring the rate of hydrolysis of S2238 measured at 405 nm. Dilutions with known amounts of purified thrombin were used for standard curves.

THROMBIN ACTIVITY ASSAY

Eckol or dieckol in 50 mM Tris–HCl buffer, pH 7.4 containing 7.5 mM EDTA and 150 mM NaCl was mixed in the absence or presence with 150 μl of AT III (200 nM). The heparins with AT III (200 nM) were dissolved in physiological saline to the appropriate concentrations and placed in the sample well. After the mixture was incubated at 37°C for 2 min, thrombin solution (150 μl; 10 U/ml) was added and incubated at 37°C for 1 min. Then, substrate for thrombin (S-2238, 150 μl; 1.5 mM) solution was added and absorbance at 405 nm was monitored for 120 s with a spectrophotometer (TECAN, Switzerland).

FACTOR Xa (FXa) ACTIVITY ASSAY

These assays were performed similar to the thrombin activity assay. Instead of thrombin and S-2238, factor Xa (1 U ml/L) and substrate S-2222 were used.

ANIMALS AND HUSBANDRY

Male ICR mice (6 weeks old upon receipt, from Orient, South Korea) were used in this study after a 12-day acclimatization period. The animals were housed as five per polycarbonate cage under controlled temperature (20–25°C) and humidity (40–45%). The light/dark cycle was 12:12 h and normal rodent pellet diet and water were supplied during acclimatization ad libitum. All animals were treated in accordance with the Guidelines for Care and Use of Laboratory Animals of Kyungpook National University.

EFFECT ON BLEEDING TIME

The tail transection bleeding time was determined according to the method of Dejana et al. [1979]. Male ICR mice were fasted overnight and eckol or dieckol was administered orally to mice. One hour after administration, the mouse tail was transected at 2 mm from the tip. Bleeding time was measured as time elapsed until bleeding stopped. When bleeding time lasted longer than 15 min, measurement was stopped and bleeding time was recorded as 15 min for statistical analyses.

ELISA FOR PAI-1 AND T-PA

The concentrations of PAI-1 and t-PA in HUVEC cultured supernatants were determined by ELISA methods, according to the manufacturer's recommended protocol.

STATISTICAL ANALYSIS

Data are expressed as the means ± SD of at least three independent experiments. Statistical significance between two groups was determined by a Student's *t*-test. The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSIONS

Phlorotannins are commonly found in brown algae and have been reported to exert various biological effects, including on antioxidant, anti-tumor, anti-cancer, and bactericidal effects and Alzheimer's disease [Nagayama et al., 2002; Okada et al., 2004; Jung et al., 2010]. In this study, the potential anticoagulant effects of purified phlorotannins (eckol and dieckol) from *E. bicyclis* were examined for the first time on the underlying mechanisms of anticoagulant activities of eckol and dieckol.

EFFECTS OF ECKOL OR DIECKOL ON aPTT AND PT

Incubation with eckol or dieckol resulted in changes of coagulation properties of human plasma. The anticoagulant properties of eckol or dieckol were tested in aPTT and PT assays using human plasma and are summarized in Tables I and II. Although the anticoagulant activities of eckol or dieckol were weaker than those of heparin, aPTT, and PT were significantly prolonged by eckol or dieckol at concentrations at or >5 μM. Prolongation of aPTT suggests inhibition of the intrinsic and/or the common pathway while prolonged PT indicates that eckol or dieckol could also inhibit the extrinsic pathway of coagulation. To confirm these in vitro data, in vivo tail bleeding time was determined. As shown in Tables I and II, tail bleeding time was significantly prolonged by eckol or dieckol with respect to the control. Surprisingly, effects of dieckol on the clotting time were better than that of eckol suggesting that hydroxyl group in eckol positively regulates the anticoagulant function of eckol (Fig. 1).

EFFECTS OF ECKOL OR DIECKOL ON THROMBIN-CATALYZED FIBRIN POLYMERIZATION

The effects of eckol or dieckol on thrombin-catalyzed fibrin polymerization in human plasma were monitored as the changes in absorbance at 360 nm as described in Materials and Methods Section. The results, presented in Figure 2, demonstrated that

TABLE I. Anticoagulant Activity of Eckol[a]

Sample	Dose	aPTT (s)	PT (s)	PT (INR)
In vitro coagulant assay				
Control	Saline	38.4 ± 1.2	16.3 ± 0.5	1.00
Eckol	0.1 μM	39.5 ± 1.7	17.0 ± 0.2	1.10
	0.5 μM	40.4 ± 1.8	17.3 ± 0.5	1.14
	1 μM	41.7 ± 0.7*	17.4 ± 1.8	1.15
	5 μM	55.9 ± 0.8**	19.5 ± 0.3*	1.48
	10 μM	71.6 ± 1.9**	26.9 ± 1.3**	3.01
	20 μM	74.3 ± 1.2**	28.0 ± 0.6**	3.29
	50 μM	75.8 ± 1.3**	28.8 ± 0.9**	3.50
Heparin		0.5 (μg/ml)	10 (μg/ml)	7.77
		108.4 ± 0.5**	41.4 ± 0.6**	

Sample	Dose	Tail bleeding time (s)	n
In vivo bleeding time			
Control	Saline	51.5 ± 7.4	3
Eckol	50 mg/kg	121 ± 3.8**	3
Heparin	50 mg/kg	165.4 ± 5.9**	3

[a]Each value represents the means ± SD (n = 5).
*$P < 0.05$ as compared to control.
**$P < 0.01$ as compared to control.

TABLE II. Anticoagulant Activity of Dieckol[a]

Sample	Dose	aPTT (s)	PT (s)	PT (INR)
In vitro coagulant assay				
Control	Saline	38.4 ± 1.2	16.3 ± 0.5	1.00
Dieckol	0.1 μM	38.5 ± 0.7	17.1 ± 0.7	1.11
	0.5 μM	40.6 ± 1.2	17.9 ± 0.9	1.23
	1 μM	51.3 ± 1.5*	17.2 ± 1.4	1.13
	5 μM	65.7 ± 0.8**	23.5 ± 0.5*	2.24
	10 μM	82.5 ± 1.4**	29.4 ± 0.7**	3.66
	20 μM	85.7 ± 0.9**	32.3 ± 1.6**	4.50
	50 μM	89.1 ± 1.2**	33.5 ± 0.7**	4.88
Heparin		0.5 (μg/ml) 108.4 ± 0.5**	10 (μg/ml) 41.4 ± 0.6**	7.77

Sample	Dose	Tail bleeding time (s)	n
In vivo bleeding time			
Control	Saline	51.5 ± 7.4	3
Dieckol	50 mg/kg	141 ± 2.5*	3
Heparin	50 mg/kg	165.4 ± 5.9**	3

[a]Each value represents the means ± SD (n = 5).
*$P < 0.05$ as compared to control.
**$P < 0.01$ as compared to control.

incubation of human plasma with both eckol and dieckol significantly decreased the maximal velocity of fibrin polymerization in human plasma. To eliminate the effect of different pH in the samples all dilutions were in 50 mM TBS, pH7.4. We also checked the effect of DMSO on human plasma at the same volume as in the case of eckol or dieckol. We did not observe any differences in coagulation properties.

EFFECTS OF ECKOL OR DIECKOL ON ACTIVITIES OF THROMBIN AND FXa

To elucidate the inhibitory mechanism of eckol or dieckol on coagulation time, their inhibitory effect on thrombin and FXa activities was measured using chromogenic substrates in the absence or presence of antithrombin III (AT III). Under the conditions of hemostatic balance the activity of thrombin is controlled by its physiological antagonist ATIII [Olds et al., 1994]. ATIII is a single-chain glycoprotein with a Mr of 58,000 Da, which is a progressive inhibitor of serine proteases [Olds et al., 1994]. The inhibitor has a binding site for heparin; its activity is increased dramatically by heparin through accelerating the binding rate to its target protease [Olson and Bjork, 1994]. ATIII interacts with several proteases of the plasma; besides thrombin, it inhibits kallikrein, factors IXa, Xa, XI, and XIIa [Davie et al., 1991; Davie, 1995]. In the absence of AT III, the amidolytic activity of thrombin was inhibited by eckol or dieckol in a dose-dependent manner, showing that the anticoagulant directly inhibited thrombin activity. However, in the presence of AT III, thrombin activity was essentially unchanged (Fig. 3A,B). Thus, AT III was unable to potentiate the activity of eckol or dieckol. Further, the effects of eckol or dieckol on FXa activity in the absence or presence of AT III were also investigated. The anticoagulant showed direct inhibitory effects on FXa activities at high concentrations, and the inhibitory effect of AT III was not changed by eckol or dieckol (Fig. 3C,D). These results were consistent with the antithrombin assay. Therefore, these results suggested that the antithrombotic mechanism of eckol or dieckol appears to be due to inhibition of fibrin polymerization and/or the intrinsic/extrinsic pathway without potentiation by AT III. Furthermore, the hydroxyl group in eckol positively regulates the anticoagulant effects on the inhibition of thrombin or FXa activity because the inhibitory effects of dieckol were better than those of eckol. The possible explanation for these results is the number and position of hydrogen donating hydroxyl groups in eckol or dieckol. Recent studies have demonstrated that the protective effect against oxidative stress induced by ROS and UV radiation is correlated with the number and position of hydrogen- donating hydroxyl groups on the aromatic ring of the phonolic molecules, and is also affected by other factors, such as other H-donating groups (–NH, –SH), etc [Rice-Evans et al., 1995; Lien et al., 1999]. Therefore, our results indicated that dieckol has more functional hydroxyl groups than eckol, therefore, this study with dieckol unravels a novel anticoagulant functions.

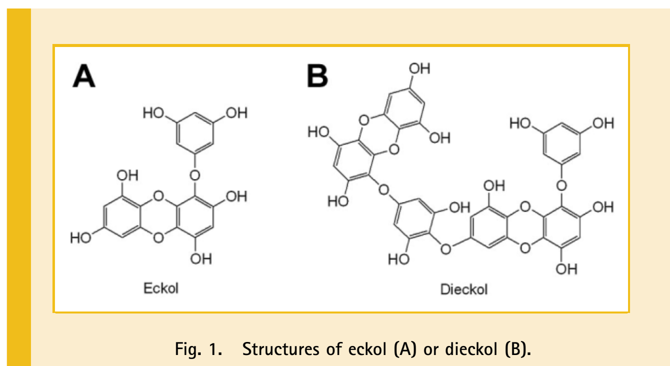

Fig. 1. Structures of eckol (A) or dieckol (B).

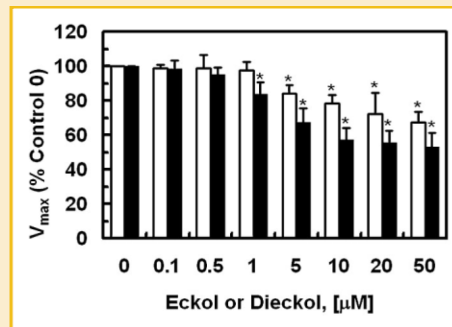

Fig. 2. Effect of eckol or dieckol on of fibrin polymerization in human plasma. Thrombin-catalyzed fibrin polymerization by indicated concentrations of eckol (□) or dieckol (■) was monitored by catalytic assay as described in Materials and Methods Section. Data represent means ± SD of three independent experiments done in triplicate. The results are measured as V_{max}, and expressed as % versus control (0 μM). *$P < 0.05$ as compared to 0.

EFFECTS OF ECKOL OR DIECKOL ON THE GENERATION OF THROMBIN AND FXa

Sugo et al. reported that endothelial cells are able to support prothrombin activation by FXa [Sugo et al., 1995]. Preincubation of FVa and FXa in the presence of $CaCl_2$ with HUVECs before addition of prothrombin resulted in thrombin generation (Fig. 4A). The effect of eckol or dieckol on thrombin generation showed that eckol or dieckol inhibited thrombin activation of prothrombin

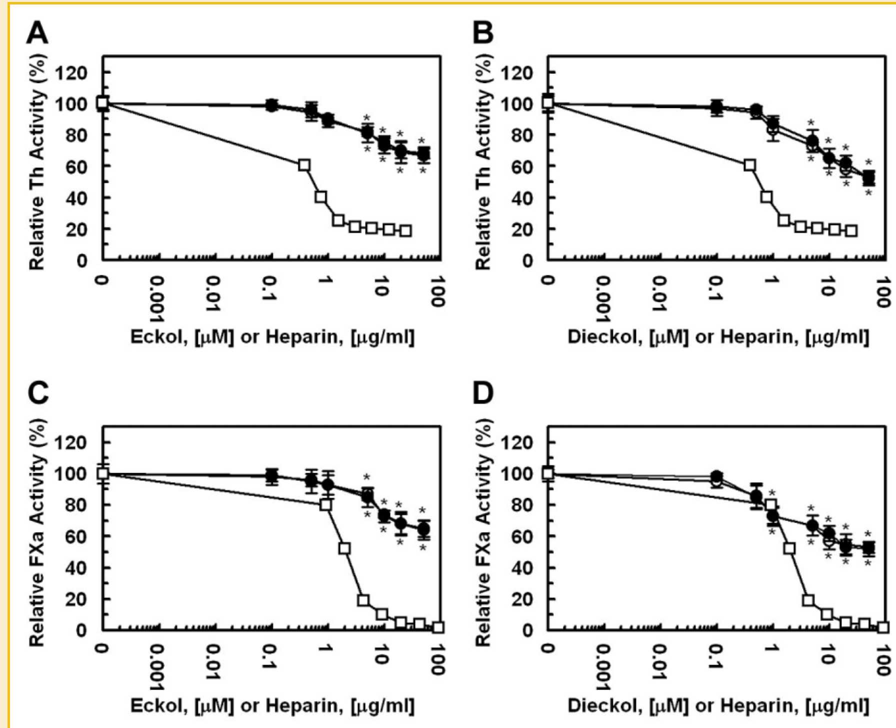

Fig. 3. Effect of eckol or dieckol on the inactivation of thrombin and factor Xa. Inhibition of thrombin (Th) in the absence of antithrombin III (○) or in the presence of antithrombin III (●) by eckol (A) or dieckol (B) was monitored by a chromogenic assay as described in Materials and Methods Section. Inhibition of factor Xa (FXa) in the absence of antithrombin III (○) or in the presence of antithrombin III (●) by eckol (C) or dieckol (D) was monitored by chromogenic assay as described in Materials and Methods Section. Heparin (□) was used as positive control. $^{*}P < 0.05$ as compared to 0.

dose- dependently (Fig. 4A). Rao et al. [1988] showed that the endothelium provides the functional equivalent of procoagulant phospholipids and supports FX activation and that in TNF-α stimulated HUVECs, FVIIa could activate FX, which was completely dependent on TF expression [Ghosh et al., 2007]. Thus, it is likely that the endothelium can provide support for FVIIa activation of FX. If so, it would be of interest to investigate the effect of eckol or dieckol on FVIIa activation of FX. HUVECs were stimulated with TNF-α to induce TF expression. As shown in Figure 4B, the rate of FX activation by FVIIa was 100-fold higher in stimulated HUVECs (56.2 ± 4 nM) compared with non-stimulated HUVECs (0.47 ± 0.3 nM), which was completely attenuated by anti-TF IgG

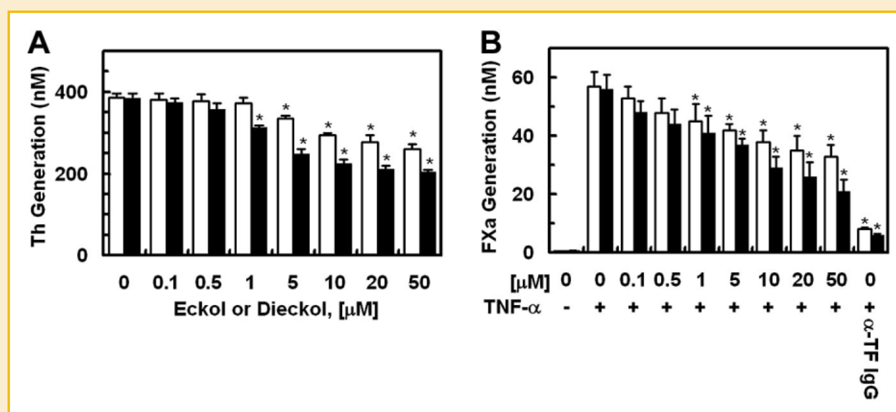

Fig. 4. Inhibition of thrombin and FXa generation by eckol or dieckol in HUVECs. A: FVa (100 pM) and FXa (1 nM) were preincubated on HUVECs monolayers for 10 min with indicated concentrations of eckol (□) or dieckol (■). Prothrombin was added to a final concentration of 1 μM and the amounts of prothrombin activated were determined at 30 min as described in Materials and Methods Section. B: HUVECs were preincubated with indicated concentrations of eckol (□) or dieckol (■) for 10 min. TNF-α (10 ng/ml for 6 h) stimulated HUVECs were incubated with FVIIa (10 nM) and FX (175 nM) in the absence or presence of anti-TF IgG (25 μg/ml) and FXa generation was then determined as described in Materials and Methods Section. $^{*}P < 0.05$ as compared to 0 (A) or TNF-α alone (B).

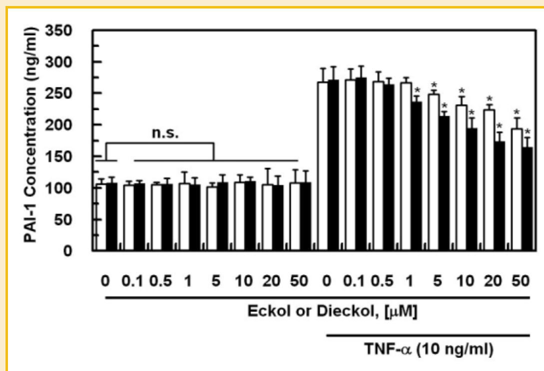

Fig. 5. Effect of various concentrations of eckol or dieckol on PAI-1 secretion in HUVECs stimulated with TNF-α. HUVECs were cultured with eckol (□) or dieckol (■) in the absence or presence of TNF-α (10 ng/ml) for 18 h and PAI-1 concentration in the culture mediums was examined as described in Materials and Methods Section. $^{*}P < 0.05$ as compared to TNF-α alone; n.s., not significant.

(6.2 ± 0.5 nM). Moreover, preincubation with eckol or dieckol dose-dependently inhibited FVIIa activation of FX (Fig. 4B). Therefore, these results suggested that eckol or dieckol could inhibit the generation of thrombin or FXa and the hydroxyl group in eckol positively regulated these functions of eckol.

EFFECTS OF ECKOL OR DIECKOL ON THE PAI-1 OR T-PA PROTEIN SECRETION

TNF-α seems to inhibit the fibrinolytic system in HUVECs via production of PAI-I and the balance between t-PA and PAI-1 is known to determine procoagulant and hypofibrinolysis activities [Schleef et al., 1988; Philip-Joet et al., 1995]. To determine the direct effects of eckol or dieckol on TNF-α stimulated PAI-1 secretion, HUVECs were cultured in media with or without eckol or dieckol in the absence or presence of TNF-α for 18 h. As shown in Figure 5,

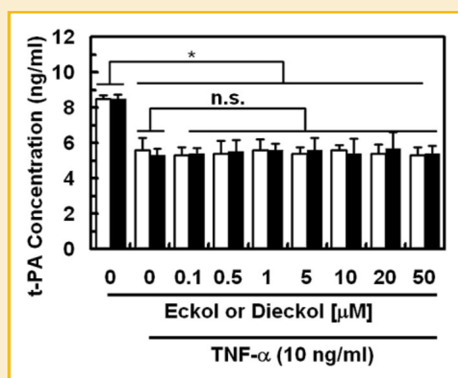

Fig. 6. Effect of various concentrations of eckol or dieckol on t-PA secretion in HUVECs stimulated with TNF-α. HUVECs were cultured with eckol (□) or dieckol (■) in the absence or presence of TNF-α (10 ng/ml) for 18 h and t-PA concentration in the culture mediums was examined as described in Materials and Methods Section. $^{*}P < 0.05$; n.s., not significant.

TABLE III. PAI-1/t-PA Ratio by Eckol or Dieckol in TNF-α Activated HUVECs by ELISA[a]

Dose		PBS	
		12.6	
		TNF-α	
10 ng/ml		51.5	
	Eckol		Dieckol
0.1 μM	51.1		50.9
0.5 μM	49.8		48.0
1 μM	47.7		42.3*
5 μM	46.1**		38.2**
10 μM	41.3**		36.1**
20 μM	38.6**		30.5**
50 μM	36.6**		28.4**

[a]Each value represents the means ± SD (n = 3).
$^{*}P < 0.05$ as compared to TNF-α alone.
$^{**}P < 0.01$ as compared to TNF-α alone.

eckol or dieckol dose dependently inhibited TNF-α induced PAI-1 secretion from HUVECs. The decrease became significant at eckol dose of 5 μM or dieckol dose at 1 μM.

Because TNF-α did not significantly affect t-PA production [Hamaguchi et al., 2003] and the balance between plasminogen activators and their inhibitors reflects the net plasminogen-activating capacity [Davie et al., 1991; Davie, 1995; Quinn et al., 2000], we investigated the effect TNF-α and eckol or dieckol on t-PA production from HUVECs. Results were consistent with the observation in which TNF-α modestly decreased t-PA production in HUVECs [Lopez et al., 2000] and this decrease was not significantly altered by treatment with eckol or dieckol (Fig. 6). Therefore, these results collectively indicate that the ratio of PAI-1/t-PA was increased by TNF-α and significantly decreased in eckol or dieckol treated conditions compared to TNF-α treated conditions (Table III).

To exclude the possibility that the inhibition of FXa/thrombin generation and profibrinolytic activities of eckol or dieckol were due to the cytotoxicity caused by eckol or dieckol treatment, MTT assays were performed in HUVECs treated with eckol or dieckol for 24 h dose dependently. Up to the concentration used (50 μM), eckol or dieckol did not affect cell viability (data not shown). Thus, the antithrombotic and profibrinolytic activities of eckol or dieckol were not due to any cytotoxic action on HUVECs.

In conclusion, this study showed that eckol or dieckol inhibited the extrinsic and intrinsic pathways of blood coagulation by inhibiting FXa and thrombin generation in HUVECs. In addition, eckol or dieckol inhibited TNF-α induced PAI-1 protein secretion. These anticoagulant and profibrinolytic effects of dieckol were better than those of eckol indicating that the number and position of hydroxyl group in eckol positively regulated anticoagulant function of eckol. These results adds to previous work and may be helpful for the rational design of pharmacological strategies for treating or preventing vascular diseases via regulation of thrombin generation.

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