Arch Pharm Res Vol 27, No 6, 619-623, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Tissue Factor Inhibitory Sesquiterpene Glycoside from *Eriobotrya japonica*

Ming Hong Lee[1], Yeon Kyoung Son[1], and Yong Nam Han[1,2]

[1]Natural Products Research Institute and [2]College of Pharmacy, Seoul National University, Seoul 110-460, Korea

(Received December 22, 2003)

Tissue factor (TF, tissue thromboplastin) is a membrane bound glycoprotein, which accelerates the blood clotting, activating both the intrinsic and the extrinsic pathways to serve as a cofactor for activated factor VII (VIIa). The TF-factor VIIa complex (TF/VIIa) proteolytically activates factors IX and X, which leads to the generation of thrombin and fibrin clots. In order to isolate TF inhibitors, by means of a bioassay-directed chromatographic separation technique, from the leaves of *Eriobotrya japonica* Lindley (Rosaceae), a known sesquiterpene glycoside (**2**) and ferulic acid (**3**) were isolated as inhibitors that were evaluated using a single-clotting assay method for determining TF activity. Another sesquiterpene glycoside (**1**) was also isolated but was inactive in the assay system. Compound **3** was yielded by alkaline hydrolysis of compound **2**. The structures of compounds **1**, **2**, and **3** were identified by means of spectral analysis as 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranosyl nerolidol (**1**), 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-[α-L-(4-trans-feruloyl)-rhamnopyranosyl-(1→6)]-β-D-glucopyranosyl nerolidol (**2**) and ferulic acid (**3**), respectively. Compounds **2** and **3** inhibited 50% of the TF activity at concentrations of 2 and 369 μM/TF units, respectively.

Key words: *Eriobotrya japonica*, Rosaceae, Sesquiterpene glycoside, Tissue factor Inhibitor

INTRODUCTION

TF is a cell surface receptor of FVII (coagulation factor VII) and is the principal initiator of the vertebrate coagulation cascade (Nemerson, 1988). Vascular damage exposes blood to cells that express TF, which causes the formation of the TF/FVII(a)/phospholipid/Ca^{2+} complex. The complex mediates the activation of both the intrinsic and the extrinsic pathways (Osterud and Rapaport, 1977). This critical position of the TF/FVIIa complex within the blood coagulation cascade makes it an attractive target for anti-thrombotic drug discovery (Harker *et al.*, 1995; Rhee and Hand, 1998; Lee *et al.*, 2002), and thus, the studies on TF inhibitors from natural products have a profound significance. We have screened various plants belonging to Rosaceae species and other plants for their inhibition on TF by measuring the prothrombin time (Lee *et al.*, 2002; Lee and Han, 2003), and the leaves of *E. japonica* showed to have the strong inhibitory activity on TF. This report describes the isolation and characterization of TF inhibitors from the leaves of this plant.

The leaves of *Eriobotrya japonica* Lindley (Rosaceae) are widely used in traditional medicine for the treatment of diabetes mellitus (Chopra and Nayar, 1965), stomachache, heatstroke, prickly heat, eczema, and various skin diseases (Shimizu *et al.*, 1986) in China and India. It is known that the leaves contain triterpenes (Liang *et al.*, 1990; De Tommasi *et al.*, 1991), sesquiterpenes (De Tommasi *et al.*, 1990; De Tommasi *et al.*, 1992), amygdalins and phenol compounds (Gray, 1972), and it is also know to have anti-oral tumor (Ito *et al.*, 2000), antiviral (De Tommasi *et al.*, 1992), anti-inflammatory properties (Shimizu *et al.*, 1986), and hypoglycemic activity (sesquiterpenes) (De Tommasi *et al.*, 1991). However, there are no previous data illustrating the TF inhibitory activity of *E. japonica*.

MATERIALS AND METHODS

Plant materials

The dried leaves of *E. japonica* were purchased from a Kyungdong market in Seoul, Korea on January 1999 and

Correspondence to: Yong Nam Han, Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea
Tel: 82-2-740-8927, Fax: 82-2-762-8322
E-mail: snake@snu.ac.kr

were verified by Emeritus Prof. H. J. Chi of Seoul National University, Korea. A voucher specimen (NPRI 990201) was deposited at the Herbarium of Natural Products Research Institute, Seoul National University, Korea.

Animals

The rats (Sprague-Dawley) were bred at the Animal Station of Natural Products Research Institute, Seoul National University. According to the Guide for the Care and Use of Laboratory Animals by Seoul National University, the rats were fed with a commercial solid food (Samyang Yuji Co. Ltd., Seoul) and tap water and were housed at 23±0.5°C and 60% humidity in a 12 h light-dark cycle.

General experimental procedures

LC-MS was measured on a Jeol Mstation JMS-700 mass spectrometer. NMR spectra were recorded on a Varian Gemini-2000 spectrometer at 300 MHz for ^{1}H-NMR and at 75 MHz for ^{13}C- NMR. Centrifugations were performed with Sorvall RT 6000 centrifuge and Sorvall OTD 65B ultracentrifuge, rotor T 865. Silica gel (63-200 μm, Merck KGaA), Sephadex LH-20 (25-100 μm, Sigma Fluka), and LiChroprep RP-18 (40-63 μm, Merck KGaA) were used for open chromatography.

Determination of tissue factor activity *in vitro*

For the single-stage clotting assay, a microsomal fraction of rat lung tissue was used as the tissue factor source (Han and Rhee, 1998). A TF stock solution from 5 g of the rat lung taken from four rats (250±10 g) contained 5.39 mg of protein/mL, where the protein concentration was determined according to Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as a standard protein. The stock solution, which was diluted 100-200 times was used for assay, which gave 5 to 10 units of TF per 100 μL. To determine the TF activity, prothrombin time was measured by the single-stage clotting assay using citrated plasma from rats (Han and Rhee, 1998; Schorer and Moldow, 1988).

The standard curve was made from the measurements of the prothrombin time that was assayed on the lung tissues from 25 rats against the pooled up plasma from nine rats. The TF activity was assessed by the single-stage clotting assay as follows: in a plastic test tube that was prewarmed in a 37°C water bath, 100 μL of plasma and 100 μL of TF solution was diluted to the proper concentration with saline and 100 μL of 25 mM $CaCl_2$ and the prothrombin time was measured after the addition of $CaCl_2$. The TF activity was considered as 100% activity when the prothrombin time with TF was 18 sec, and the plasma recalcification time without TF was considered as 0% activity. The activity of TF that gave 50% acceleration of the prothrombin time on the standard curve was arbitrarily defined as one unit of TF. The inhibition percentage was obtained by calculation of the decrease extent of TF activity in a unit from the standard curve. The IC_{50} value of the TF inhibitor was obtained from a logit-log graph (a $\%B/B_o$-log graph) pertaining to the relationship between the inhibition percentage and the concentration of the inhibitor in log scale.

Extraction and isolation

The dried leaves of *E. japonica* (20 kg) were percolated with MeOH. The methanolic extract (2.6 kg) was successively partitioned between $CHCl_3$, *n*-BuOH, and H_2O to afford 446, 720, and 1420 g of residues, respectively. Chromatography of the *n*-BuOH fraction over the silica gel eluted with a $CHCl_3$ MeOH gradient (10:1, 5:1, 3:1, 1:1, MeOH) afforded an active fraction A (230 g, $CHCl_3$ MeOH/5:1-3:1). The fraction A was subjected to column chromatography on a silica gel eluted with a $CHCl_3$-MeOH-H_2O gradient (6:1:0.1, 3:1:0.1, MeOH) to give an active sub-fraction A_1 (26 g, $CHCl_3$-MeOH-H_2O/3:1:0.1). Chromatography of fraction A_1 on Sephadex-LH 20 (50% MeOH) was performed to give an active sub-fraction A_2 (4 g, 250-350 mL), which was further chromatographed on the reverse phase C-18 (70% MeOH, 120-180 mL) to yield compounds **1** (450 mg) and **2** (900 mg).

Compound 1

(De Tommasi *et al.*, 1990), white powder, ^{1}H-NMR (300 MHz, CD_3OD) δ_H (ppm): 5.21 (1H, d, *J*=11.0 Hz, H-1a), 5.22 (1H, d, *J*=18.0 Hz, H-1b), 5.92 (1H, dd, *J*=18.0, 11.0 Hz, H-2), 1.96-2.08 (2H, m, H-4), 1.96-2.08 (2H, m, H-8), 1.60 (3H, s, H-12), 1.37 (3H, brs, H-13), 1.59 (3H, brs, H-14), 1.66 (3H, brs, H-15), 4.41 (1H, d, *J*=7.2 Hz, glc H'-1), 5.15 (1H, d, *J*=1.2 Hz, rha H''-1), 1.25 (1H, d, *J*=6.3 Hz, rha H''-6), 5.31 (1H, d, *J*=1.2 Hz, rha H'''-1), 1.27 (1H, d, *J*=6.3 Hz, rha H'''-6), 4.71 (1H, d, *J*=1.2 Hz, rha H-1), 1.23 (1H, d, *J*=6.3 Hz, rha H''''-6); ^{13}C- NMR (75 MHz, CD_3OD) δ_C (ppm): 115.8 (C-1), 144.6 (C-2), 81.9 (C-3), 42.7 (C-4), 25.9 (C-5), 125.6 (C-6), 136.2 (C-7), 40.8 (C-8), 27.9 (C-9), 125.7 (C-10), 132.0 (C-11), 16.2 (C-12), 22.8 (C-13), 18.8 (C-14), 23.6 (C-15), 98.2 (glc' C-1), 81.7 (glc' C-2), 77.7 (glc' C-3), 72.0 (glc' C-4), 76.4 (glc' C-5), 68.0 (glc C-6), 103.5 (rha C''-1), 72.5 (rha C''-2), 72.2 (rha C''-3), 79.9 (rha C''-4), 68.1 (rha C''-5), 17.9 (rha C''-6), 101.4 (rha C'''-1), 72.5 (rha C'''-2), 73.0 (rha C'''-3), 73.8 (rha C'''-4), 69.8 (rha C'''-5), 18.1 (rha C'''-6), 101.9 (rha C''''-1), 72.6 (rha C''''-2), 72.3 (rha C''''-3), 74.1 (rha C''''-4), 70.5 (rha C''''-5), 17.8 (rha C''''-6).

Compound 2

(De Tommasi *et al.*, 1992), yellow powder, LC-MS *m/z*: 997 $[M-H]^+$; ^{1}H-NMR (300 MHz, CD_3OD) δ_H (ppm): 5.21

(1H, dd, *J*=10.8, 1.0 Hz, H-1a), 5.23 (1H, dd, *J*=17.7, 1.0 Hz, H-1b), 5.94 (1H, dd, *J*=17.7, 10.8 Hz, H-2), 1.96-2.09 (2H, m, H-4), 1.96-2.09 (2H, m, H-8), 1.58 (3H, brs, H-12), 1.38 (3H, brs, H-13), 1.56 (3H, brs, H-14), 1.64 (3H, brs, H-15), 4.43 (1H, d, *J*=7.2 Hz, glu H'-1), 5.15 (1H, d, *J*=1.2 Hz, rha H"-1), 1.28 (1H, d, *J*=6.3 Hz, rha H"-6), 5.34 (1H, d, *J*=1.2 Hz, rha H'"-1), 1.24 (1H, d, *J*=6.0 Hz, rha H'"-6), 4.78 (1H, d, *J*=1.5 Hz, rha H""-1), 1.18 (1H, d, *J*=6.0 Hz, rha H""-6), 7.64 (1H, d, *J*=15.6 Hz, Feru α-H), 6.38 (1H, d, *J*=15.6 Hz, Feru β-H), 7.18 (1H, d, *J*=1.8 Hz, Feru 2-H), 6.81 (1H, d, *J*=8.1 Hz, Feru 5-H), 7.07 (1H, dd, *J*=8.1, 1.8 Hz, Feru 6-H), 3.89 (3H, s, Feru OCH_3); ^{13}C- NMR (75 MHz, CD_3OD) δ_C (ppm): 115.7 (C-1), 144.7 (C-2), 82.0 (C-3), 42.7 (C-4), 25.9 (C-5), 125.6 (C-6), 136.2 (C-7), 40.8 (C-8), 27.8 (C-9), 125.6 (C-10), 132.0 (C-11), 16.3 (C-12), 22.8 (C-13), 18.8 (C-14), 23.6 (C-15), 98.1 (glu' C-1), 81.6 (glu' C-2), 77.7 (glu' C-3), 72.3 (glu' C-4), 76.2 (glu' C-5), 67.8 (glu' C-6), 103.6 (rha C"-1), 72.6 (rha C"-2), 72.3 (rha C"-3), 79.9 (rha C"-4), 68.1 (rha C"-5), 17.9 (rha C"-6), 101.4 (rha C'"-1), 72.5 (rha C'"-2), 73.0 (rha C'"-3), 73.8 (rha C'"-4), 71.8 (rha C'"-5), 18.1 (rha C'"-6), 102.1 (rha C""-1), 72.6 (rha C""-2), 72.5 (rha C""-3), 75.5 (rha C""-4), 70.5 (rha C""-5), 17.9 (rha C""-6), 169.0 (Feru COO-), 147.1 (Feru C-α), 116.6 (Feru C-β), 127.5 (Feru C-1), 111.7 (Feru C-2), 149.5 (Feru C-3), 149.5 (Feru C-4), 115.3 (Feru C-5), 124.2 (Feru C-6), 56.5 (Feru OCH_3).

Alkaline hydrolysis of compound 2 was performed using standard procedures to yield ferulic acid (**3**). It was identified through the comparison with an authentic sample.

Statistical analysis

The results of IC_{50} values were expressed as means± S.D. from three separated experiments. The student's *t*-test was used to evaluate the differences in the means between compounds **2** and **3**, accepting the *p* value < 0.0001 as significant.

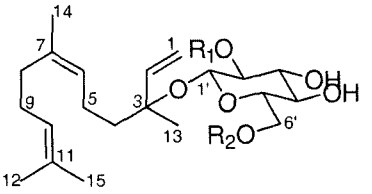
RESULTS

A variety of assay methods for measuring tissue factor (TF) activity has been developed (Schorer and Moldow, 1988). We found the single-stage clotting assay of total procoagulant activity in rat tissues to be simple and reproducible (Han and Rhee, 1998; Schorer and Moldow, 1988). Activity-guided isolation of the BuOH fraction yielded compound **2** as a potent TF inhibitor, while compound **1** was inactive in the assay system (Table I). Compounds **1** and **2** have been previously isolated from the leaves of this plant (De Tommasi *et al.*, 1990; 1992), and their structures (Chart 1) were identified through a comparison of spectra data of ^1H-NMR, ^{13}C-NMR, DEPT, and MS with De Tommasi *et al.* published values (De Tommasi *et al.*, 1990 and 1992).

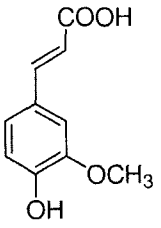
Table I. IC_{50} values for fractions and compounds from *E. japonica*

Fraction	Weight (g)	IC_{50}/TF unit*
MeOH ex.	2600	370 ± 20 μg**
BuOH ex.	720	17.8 ± 5.0 μg
Fr. A	230	11.1 ± 2.0 μg
Fr. A_1	26	5.4 ± 1.0 μg
Fr. A_2	4	1.3 ± 0.3 μg
1		Inactive
2		2 ± 0.2 μM
3		369 ± 51 μM

* The activity of TF that accelerated 50% the prothrombin time was arbitrarily defined as one unit of TF, when the prothrombin time was determined by the single-stage clotting assay.
** means ± SD (n=3)



1 : R_1=Rha(1''-4''')Rha, R_2=Rha(''''')
2 : R_1=Rha(1''-4''')-Rha, R_2=(4''''-*trans*-feruloyl)-Rha
Rha=α-L-rhamnopyranosyl



3: Ferulic acid

Fig. 1. Structures of compound **1**, **2**, and **3**

Prothrombin time elongation by 2 and 3

Fig.1 showed that compounds **2** and **3** did not alter the plasma recalcification time in the absence of TF, but instead, elongated the prothrombin time in the presence of TF in a dose-dependence manner. The inhibition percentages of compounds **2** and **3** on TF activity, as shown in Fig. 2, were obtained by calculating the decrease extent of TF activity in units from the data under the presence of 6.5 units of TF as shown in Fig. 1.

TF inhibitory activity and SAR analysis

The TF inhibitory activities of compound **2** and ferulic acid isolated from *E. japoninca* were summarized in Table I. Compound **2** (IC_{50}= 2 μM/TF unit) was found to be more potent than ferulic acid (IC_{50}= 369 μM/TF unit). Compound **1**, which is deficient in feruloyl moiety of compound

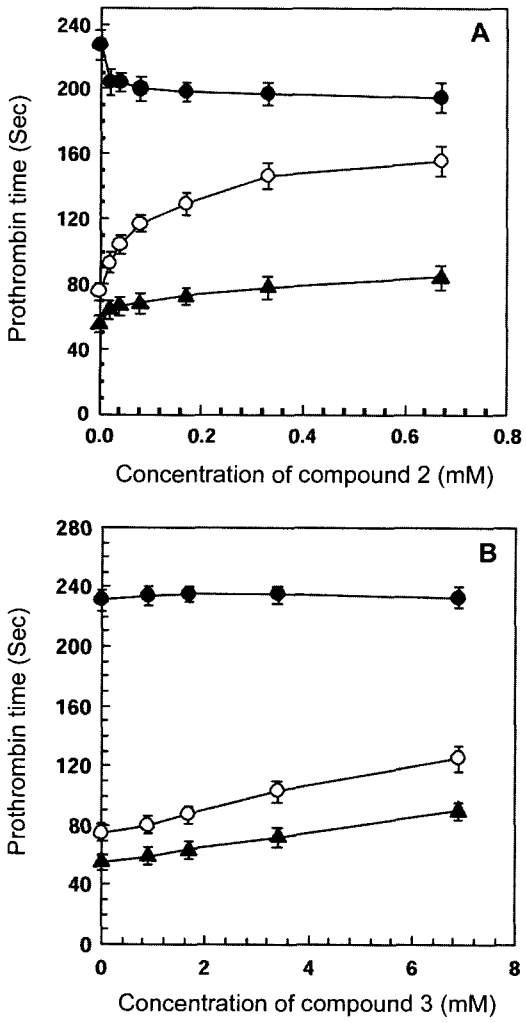


Fig. 1. Prothrombin time elongation by compounds **2** (A) and **3** (B) in the presence of rat lung tissue factor. A mixture of 360 μL of TF solution and 40 μL of sample solution in 0.1 M Tris buffer (pH 7.4) was preincubated at 37°C for 20 min, and an aliquot (100 μL) of the mixture was added to 100 μL of plasma that was prewarmed at 37°C, and the prothrombin time was measured after the addition of 100 μL of 25 mM $CaCl_2$. Curve ●: the plasma recalcification time without the addition of TF solution to plasma (control). Curves ○ and ▲ : the prothrombin time with the addition of 6.5 and 13.0 units of TF solution to plasma, respectively. Means ± S.D. In the situation of lower TF units and higher concentration of inhibitor the PT will prolongate longer.

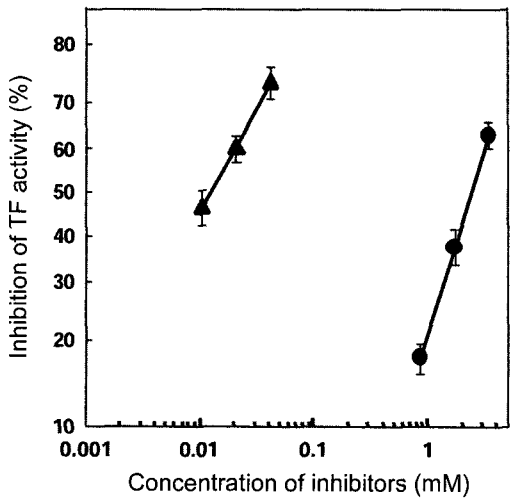


Fig. 2. The inhibition percentage of compounds **2** (▲) and **3** (●) on tissue factor activity. The percentage was calculated by the decrease extent of TF activity in units that was obtained from the data under the presence of 6.5 units of TF as shown in Fig. 1 and as detailed under the experimental section. Means ± S.D. and *t*-test significance levels were calculated with relative values. **P*<0.0001 compared the TF inhibitory extent of compounds **2** and **3**.

2, did not show any TF inhibitory activity. These results indicated that the ferulic acid moiety in compound **2** play a pivotal role in TF inhibitory activity, and the sesquiterpene (nerolidol) moiety might have a latent effect on TF.

DISCUSSION

The TF inhibitory activity of compounds **1**, **2**, and **3** was assessed by the prolongation of the prothrombin time, which was measured by using a onestage clotting assay *in vitro* (Han and Rhee, 1998; Lee and Han, 2003). The correlation between inhibition percentage and concentration was drawn on a logit-log graph as depicted in Fig. 2. The results indicated that compound **2** showed a strong TF inhibitory activity (IC_{50}, 2 μM) while compound **3** illustrated a weak activity (IC_{50}, 369 μM), and compound **1** did not show any TF inhibitory activity (Table I).

Interestingly, compound **2** is composed of the three parts: nerolidol, carbohydrate, and feruloyl moieties, and showing the strong TF inhibitory activity. Whereas compound **1**, without the feruloyl moiety, did not show any TF inhibitory activity, while ferulic acid (compound **3**) only showed a weak activity. These results suggest that the ferulic acid moiety in compound **2** play a pivotal role in TF inhibitory activity, and that the sesquiterpene (nerolidol) moiety can increase the inhibitory activity against TF. Wang *et al.* (1998) reported that anacardic acids, the unsaturated fatty acid derivatives at the 6 position of salicylic acid, showed signs of inhibitory activity (IC_{50}, 30~80 μM) against soluble TF/VIIa, and the fatty acid moiety that contained two or three double bonds maight exert their activity by binding to VIIa, and consequently preventing the binding of sTF to VIIa. The structure of nerolidol (sesquiterpene) having three double bonds is similar to that of the unsaturated fatty acid. This implied the nerolidol part may exert its latent effect on TF in compound **2**. Compound **2** as a new inhibitory agent provides a drug candidate for regulating blood coagulation.

Recently, a new TF inhibitory triterpene (2α,3β-dihydroxyolean-12-ene-24,28-dioic acid) named chaenomelogenin

A (IC_{50}, 28 μM) was isolated from the fruits of *Chaenomeles sinensis* (Lee and Han, 2003). It contained carboxyl and lipophilic olefine groups. All the compounds showing the TF inhibitory activity described above have the carboxyl and lipophilic olefine groups. It may be possible to design more effective inhibitors based on these evidence.

ACKNOWLEDGEMENTS

We are grateful to Emeritus Prof. H. J. Chi, Natural Products Research Institute, Seoul National University for the identification of the plant material. This research was supported by a grant (01-PJ2-PG6-01NA01-0002) from the Ministry of Health and Welfare, ROK.

REFERENCES

Chopra, R. N. and Nayar, S. L., Glossary of Indian Medicinal Plants, C.S.I.R., New Dehli, p. 168 (1965).

De Tommasi, N., Aquino R., De Simone, F., and Pizza, C., Plant metabolites. New sesquiterpene and ionone glycosides from *Eriobotrya japonica*. *J. Nat. Prod.*, 55, 1025-1032 (1992).

De Tommasi, N., De Simone, F., Aquino, R., and Pizza, C., Plant metabolites. New sesquiterpene glycosides from *Eriobotrya japonica*. *J. Nat. Prod.*, 53, 810-815 (1990).

De Tommasi, N., De Simone, F., Cirino G., Cicala C., and Pizza, C., Hypoglycemic effects of sesquiterpene glycosides and polyhydroxylated triterpenoids of *Eriobotrya japonica*. *Planta Med.*, 57, 414-416 (1991).

De Tommasi, N., De Simone, F., Pizza, C., Mahmood, N., Moore, P. S., Conti, C., Orsi, N., and Stein M. L., Constituents of *Eriobotrya japonica*. A study of their antiviral properties. *J. Nat. Prod.*, 55, 1067-1073 (1992).

Gray, D. O., Isolation of 4-methylene-DL-proline from *Eriobotrya japonica*. *Phytochem.*, 11, 745-750 (1972).

Han, Y, N. and Rhee, I. K., Changes of tissue factor activity on inflammatory stimulus and aging in rat. *Arch. Pharm. Res.*, 21, 549-554 (1998).

Harker, L. A., Hanson, S. R., and Kelly, A. B., Antithrombotic benefits and hemorrhagic risks of direct thrombin antagonists. *Thromb. Haemost.*, 74, 464-472 (1995).

Ito, H., Kobayashi, E., Takamatsu, Y., Li, S. H., Hatano, T., Sakagami, H., Kusama, K., Satoh, K., Sugita, D., Shimura, S., Itoh, Y., and Yoshida, T., Polyphenols from *Eriobotrya japonica* and their cytotoxicity against human oral tumor cell lines. *Chem. Pharm. Bull.*, 48, 687-693 (2000).

Lee, M. H. and Han, Y. N., A new *in vitro* tissue factor inhibitory triterpene from the fruits of *Chaenomeles sinensis*. *Planta Med.*, 69, 327-331 (2003).

Lee, M. H., Son, Y. K., and Han, Y. N., Tissue factor inhibitory flavonoids from the fruits of *Chaenomeles sinensis*. *Arch. Pharm. Res.*, 25, 842-850 (2002).

Liang, Z. Z., Aquino, R., Feo V. D., Simone F. D., and Pizza C., Polyhydroxylated triterpenes from *Eriobotrya japonica*. *Planta Med.*, 56, 330-332 (1990).

Lowry, O. H., Roseburgh, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Foline phenol reagent. *J. Biol. Chem.* 193, 265-275 (1951).

Nemerson, Y., Tissue factor and hemostasis. *Blood*, 71, 1-8 (1988).

Osterud, B. and Rapaport S. I., Activition of factor IX by the reaction product of tissue factor and factor VII: Additional Pathway for initiating blood coagulation. *Pro. Natl. Acad. Sci.* U. S. A., 74, 5260-5364 (1977).

Schorer, A. E. and Moldow, C. F., Chapter 6. Production of tissue factor. In Ryan, U.S. (Ed). Endothelial Cells. Vol. I. CRC Press, Boca Raton, Florida, pp. 85-105 (1988).

Shimizu, M., Fukumura, H., Tsuji, H., Tanaami, S., Hayashi, T., and Morita, N., Anti-inflammatory constituents of topically applied Crude Drugs. I. Constituents and anti-inflammatory effect of *Eriobotrya japonica* Lindley. *Chem. Pharm. Bull.*, 34, 2614-2617 (1986).

Wang, D., Girard, T. J., Kasten T. P., LaChance, R. M., Miller-Wideman, M. A., and Durley, R. C., Inhibitory activity of unsaturated fatty acids and anacardic acids toward soluble tissue factor-factor VIIa complex. *J. Nat. Prod.*, 61, 1352-1355 (1998).