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# Identification of a Neolignan Glycoside from the Pine Tree, *Pinus densiflora* Showed Antithrombotic Activity

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The constituents from the needles of the pine tree, *Pinus densiflora*, were purified and investigated for antithrombotic activity. The needles were initially extracted three times with 70% ethanol, and the extract was sequentially fractionated with chloroform and *n*-butanol. The aqueous layer formed after *n*-butanol fractionation was subjected to purification by medium pressure and high pressure liquid chromatography. The two neolignans, 2, 3-dihydro - 7 - hydroxyl - 3 - hydroxymethyl - 2-(4´-hydroxyl - 3-methoxyphenyl) - 5 - benzofuranpropanol - 3 - *O* - α-rhamnopyranoside (a neolignan glycoside) and 2, 3-dihyro-3-hydroxymethyl-7-methoxy-2-(4´-hydroxyphenyl-3´-methoxy)-5-benxofuran propanol 4´-*O*-α-rhamnopyranoside (icariside $E_4$) were identified by $^{1}H$ and $^{13}C$ NMR spectra. The effect of the purified compounds, the neolignan glycoside and icariside $E_4$ on thrombin inhibition were investigated by measuring thrombin clotting time in plasma. As a result, the clotting of the neolignan glycoside was delayed four times compared to that of icariside $E_4$. In addition, an analysis of the inhibition effect by changing the concentration showed that the clotting time was delayed in accordance with an increase in the concentration of the neolignan glycoside. Furthermore, we examined the interaction of thrombin and fibrinogen to clarify the action mechanism. As a result, the delay of clotting time in the response of thrombin and pure fibrinogen may indicate that neolignan glycosides inhibit the thrombin action in a direct manner, leading to the suppression of fibrin generation.

**Key words** : Antithrombotic agent, fibrin, neolignan glycoside, *Pinus densiflora*, thrombin

## Introduction

A diversity of lifestyles, such as changes in eating habits and lack of exercise, along with rapid economic growth, has caused habitual diseases such as cardiovascular disease and stroke, which have become a major cause of morbidity and mortality. Although many pathophysiological processes are associated with the chronic development of cardiovascular disease, thrombosis is one of the causes triggering stroke and acute coronary syndrome. Although anti-platelet agents including aspirin and anticoagulant agents such as heparin and warfarin are candidates for treating thromboembolic diseases, these drugs show many potential limitations. In particular, major ischemic cardiovascular diseases frequently accompany these diseases, and bleeding complications represent a major disadvantage [5, 12, 20]. The contribution of protease-activated receptors (PARs) to coagulation has also been investigated *in vivo* using antagonists to PAR-1 [24]. Thrombin is a serine protease catalyzing cleavage of fibrinogen and other clotting factors, leading to activation of the coagulation system and the platelet response. This reaction allows thrombin to play an integral role in hemostasis and thrombosis. The constituents from *Pinus densiflora* Sieb et Zucc (Pinaceae). have been purified in search of potential candidates for an antithrombotic agent. *P. densiflora*, is found abundantly in mountainous regions of Korea, Japan, and China [12]. Pine needles have been traditionally used in Korean medicine for suppressing liver and gastroenteric problems, as well as skin diseases [10, 11, 12, 16]. The chemical constituents, including essential oils (α-pinene, β-pinene, camphene, phellandrene, limonene, borneol, and bornyl acetate), cinnamic acid, and benzoic acid have thus far been reported in the *Pinus* genus [13, 14, 15]. Previous studies have reported chemical constituents with antioxidant activity, including 5,7,8,4′-tetrahydroxy-3-methoxy-6-methylflavone-8-*O*-β-D-glucopyranoside, kaempferol 3-*O*-galactopyranoside and its 6″-acetate, (+)-isolarisiresinol xylopyranoside, and (+)-catechin [1, 7, 8].

In this study, two unique constituents from *P. densiflora*

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were newly identified and investigated for a putative antithrombotic agent candidate.

## Materials and Methods

### Plant, chemicals and instruments

*P. densiflora* needles were collected at Youngwol, Gangwondo, Korea in April 2012 and were authenticated by the Korea Environmental and Ecological Services. Plasma (bovine) and fibrinogen (Type 1-s, from bovine plasma) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and thrombin (500 NIH U/ml) was supplied by Mochida Pharmaceutical Co. (Tokyo, Japan). Heparin (Sigma) was used as a control and Dade® Owren's veronal buffer (pH 7.25±0.1) solution (Bohemia, NY, USA) was also used in this work. Unless specified, other chemicals were of analytical grade.

HPLC analysis was carried out on a Gilson model GX-271 liquid handler system (Gilson, MD, USA). The mobile phase was 43% methanol and C18-10E Shodex packed column (250×10 mm i.d., 50, 100Å) equipped with a Shodex RI-71 detector were used. The optical rotation was estimated using a JASCO DIP-370 digital polarimeter (1% $CH_3OH$, cell length ; 100 mm, volume; 1 ml, Easton, MD, USA).

$^1H$ and $^{13}C$ NMR spectra were measured by Varian Unity INOVA 500 instruments (Palo Alto, CA, USA). $CD_3OD$ was used as the solvent, and the chemical shift expressed in ppm was confirmed by the solvent peak (δH 3.30 ppm, δC 49.0 ppm).

### Extraction and purification

The diagrammatic scheme for the extraction of constituents from *P. densiflora* is shown in Fig. 1. The powdered needles of *P. densiflora* (2.4 kg) were refluxed in 70% ethanol for 3 hr (20 l × 3 times). The total filtrate was concentrated to dryness *in vacuo* at 40°C to render the ethanol extract. The extract was then suspended in 10 volumes of 5% methanol and partitioned with an equal volume of chloroform. Each partitioned solvent was concentrated *in vacuo* and the resulting extracts were analyzed for antithrombotic activity. The aqueous part of the *n*-butanol fraction, showing the highest thrombin inhibition activity, was chromatographed on C-18 silica gel medium-pressure liquid chromatography. The *n*-butanol fraction was eluted with mobile phase of 20, 40, 60, 80, and 100% methanol sequentially to yield 15 fractions (B1~B15). Thrombin inhibition activities of the isolated

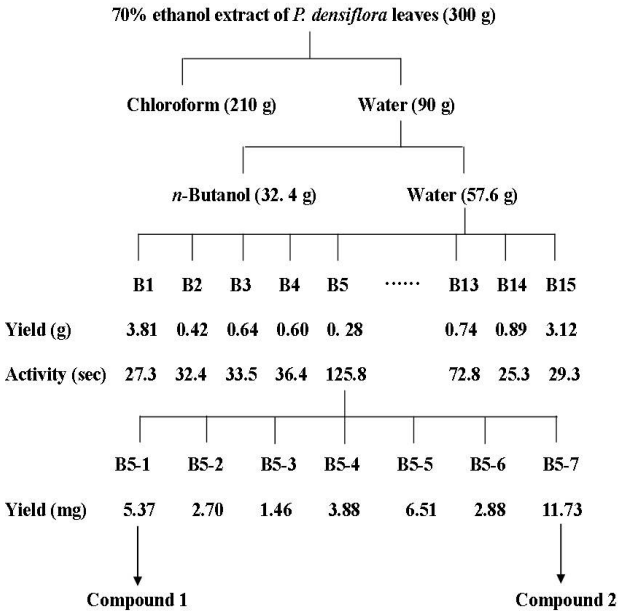


Fig. 1. Schematic diagram for extracting the *Pinus densiflora* constituents. Fractionation was guided by a thrombin inhibition assay.

fractions were measured, and the B5 fraction representing the highest activity was separated by HPLC to yield seven fractions (B5-1~B5-7). Constituents corresponding to the main peak, the B5-1 (compound 1) and B5-7 (compound 2) fractions, were structural analyzed for further study.

### Antithrombotic activity assay

Antithrombotic activity measured APTT (activited partial thromboplastin time), PT (prothrombin time) and TT (thrombin time). APTT and PT were measured using a Dade® actin and a Dade® thromboplastin C plus, respectively. Thrombin time was measured by the slight change in turbidity using the method of Born and Cross [15] and a coagulometer (KC-A1, Amelung, Japan). In a tube, 50 µl 0.5 U thrombin and 50 µl 20 mM $CaCl_2$ were mixed with 10 µl of the purified compounds dissolved in DMSO and DMSO (control), and pre-incubated for 2 min at 37°C. Plasma clotting was determined by measuring the time consumed after adding and mixing 100 µl plasma [21]. A 50 µl aliquot of 5 U thrombin and 50 µl of 20 mM $CaCl_2$ were mixed with 10 µl of the purified compounds dissolved in DMSO and DMSO (control), in a coagulometer tube and pre-incubated for 2 min at 37°C. Fibrinogen clotting was determined by estimating the time consumed after adding and mixing 100 µl of 0.33% fibrinogen in the tube.

## Results and Discussion

### Structure of compound 1

The chemical shift values should show in parenthesis, as report of Nakanishi [21]. A methoxy group was bonded to the C3′ residue in the heteronuclear multiple bond correlation (HMBC) and nuclear overhauser effect spectroscopy (NOESY) data (Table 1). In addition, a proton peaks originating from a hydrofuran ring were identified in δ 5.44 (1H, d, *J* = 6.5 Hz, H-2), δ 3.59 (1H, overlapped, H-3), δ 3.85 (1H, dd, 10.8, 8.0 Hz, H-3a) and δ 3.71 (1H, dd, *J* = 10.5, 5.0 Hz, H-3a), indicating the presence of a benzofuran backbone. Three methylene proton peaks originating from the hydroxypropyl group were observed in δ 2.56 (2H, t, *J* = 7.5 Hz, H-5a), 1.77 (2H, tt, *J* = 7.5, 6.5 Hz, H-5b) and 3.53 (2H, t, *J* = 6.5 Hz, H-5c), and were connected to the C5 position of benzene ring B in the HMBC spectrum. Moreover, the $^{1}H$ and $^{13}C$ NMR spectral analysis suggested that compound 1 carried α-rhamnose. Its relation to the anomeric proton of rhamnose on the HMBC and NOESY spectra indicated that α-rhamnose was connected to C-3a of benzofuran. These findings suggest that compound 1 was a type of neolignan containing a 2,3-dihydro-3-hydroxylmethyl-2-5-benzofuranpropanol backbone, which was previously reported in *Larix leptoleps* (*Pinus massoniana*), and *Juniperus communis* [21]. Optical rotation was estimated at a $[\alpha]_D$ value of -9.7. These results demonstrate that compound 1 was identified and characterized as (2*S*, 3*R*)-2,3-dihydro-7-hydroxy- 3-hydroxymethyl-2-(4′-hydroxy-3′-methoxyphenyl)-5-benzofuranpropanol 3a-*O*-α-rhamnopyranoside (a neolignan glycoside), which was homologous to the structure and optical rotation value isolated from *Juniperus communis* [18, 21]. (Fig. 2A).

Table 1. $^{1}H$- and $^{13}C$-NMR data of compound 1[a)]

| Position | $^{1}H$ (δ, ppm) | $^{13}C$ (δ, ppm) |
|---|---|---|
| 2 | 5.44, d (*J*=6.5) | 89.2 |
| 3 | 3.59, overlapped | 53.0 |
| 3a | 3.85, dd (*J*=10.8, 8.0) | 70.4 |
| | 3.71, dd (*J*=10.5, 5.0) | - |
| 4 | 6.56, brs | 116.4 |
| 4a | - | 129.4 |
| 5 | - | 136.8 |
| 5a | 2.56, t (*J*=7.5) | 32.7 |
| 5b | 1.77, tt (*J*=7.5, 6.5) | 35.6 |
| 5c | 3.53, t (*J*=6.5) | 62.2 |
| 6 | 6.57, brs | 116.1 |
| 7 | - | 141.9 |
| 7a | - | 147.4 |
| 1′ | - | 134.7 |
| 2′ | 6.93, d (*J*=1.5) | 110.4 |
| 3′ | - | 149.1 |
| 4′ | - | 146.4 |
| 5′ | 6.76, d (*J*=8.5) | 117.2 |
| 6′ | 6.82, dd (*J*=8.5, 1.5) | 119.8 |
| 3′ $OCH_3$ | 3.82, s | 56.4 |
| Rha 1′′ | 4.75, d (*J*=1.5) | 101.7 |
| 2′′ | 3.80, dd (*J*=1.5, 3.0) | 72.2 |
| 3′′ | 3.59, dd (*J*=3.0, 9.5) | 72.5 |
| 4′′ | 3.35, t (*J*=9.5, 10) | 73.8 |
| 5′′ | 3.53, overlapped | 70.2 |
| 6′′ | 1.24, d (*J*=6.5) | 18.0 |

a) Measured in $CD_3OD$ (500 MHz for $^{1}H$ and 125 MHz for $^{13}C$)
*Assignment based on the HSQC and HMBC data.

### Structure of compound 2

Analysis of compound 2 by 1D and 2D NMR spectral data indicated that its chemical structure was similar with that of compound 1. Three proton peaks of 1′,3′,4′,-trisubstituded benzene ring (ring A) were observed in δ 7.03 (1H, d, *J* = 2.0 Hz, H-2′), δ 7.06 (1H, d, *J* = 8.5 Hz, H-5′) and δ 6.89 (1H, dd, *J* = 8.5, 2.0 Hz, H-6′), and two proton peaks of tetrasubstituded benzene ring (ring B) were measured at δ 6.71 (1H, brs, H-4) and δ 6.72 (1H, brs, H-6), respectively. In addition, three methylene proton peaks based on the hydroxypropyl group were observed in δ 2.60 (2H, t, *J* = 7.5 Hz, H-5a), δ 1.78 (2H, tt, *J* = 7.5, 6.5 Hz, H-5b) and δ 3.54 (2H, t, *J* = 6.5 Hz, H-5c). The proton peaks from the hydrofuran ring were also observed in δ 5.54 (1H, d, *J* = 6.0 Hz, H-2), δ 3.44 (1H, overlapped, H-3), δ 3.77 (1H, dd, *J*=10.6,

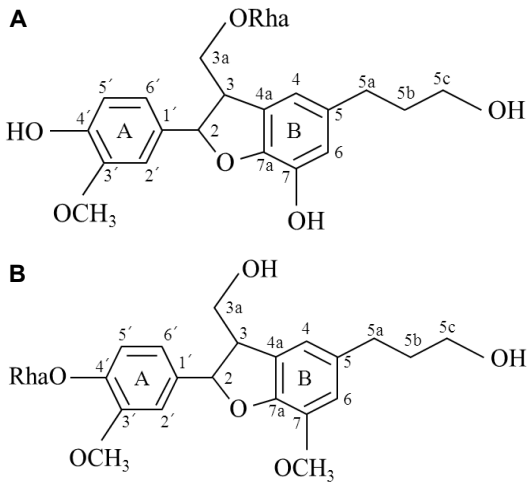


Fig. 2. Chemical structures of the purified neolignan glycoside (A) and icariside E4 (B) from *Pinus densiflora*.

8.0 Hz, H-3a) and δ 3.86 (1H, dd, *J*=10.5, 5.0 Hz, H-3a). This finding indicates that the chemical structure of compound 2 contained a benzofuran backbone, and that a hydroxypropyl group was present in its C5 position. In addition, a couple of methyl protons at δ 3.85 and δ 3.78 of HMBC spectrum have a relationship with H-2′ and H-6′/C-2, and H-4 and H-6/C-5a, respectively. This may indicate that the two methyl protons are connected with C7 and C3′. Similar to compound 1, compound 2 carried α-rhamnose based on the $^{1}H$ and $^{13}C$ NMR spectral data but the HMBC and NOESY spectra suggested that α-rhamnose was bonded to the C4′ position of ring A. This kind of chemical structure has been reported in about 20 species of plants, including *Picea abies, Betula pendul,* and *J. communis* [5, 17, 19, 21, 25, 26]. The optical rotation of compound 2 was $[\alpha]_D$ -27, suggesting that it consisted of a homologous chemical structure found in *J. communis* study [21], (2*R*,3*S*)-2,3-dihyro-3-hydroxymethyl-7-methoxy-2-(4′-hydroxyphenyl-3′-methoxy)-5-benxofuran propanol 4′-*O*-α-rhamnopyranoside (icariside $E_4$). The physical data and chemical structure of this compound is shown in Table 2 and Fig. 2B.

Table 2. $^{1}H$- and $^{13}C$-NMR data of compound 2[a)]

| Position | $^{1}H$ (δ, ppm) | $^{13}C$ (δ, ppm) |
|---|---|---|
| 2 | 5.54, d (*J*=6.0) | 89.2 |
| 3 | 3.44, overlapped | 53.0 |
| 3a | 3.77, dd (*J*=10.6, 8.0) | 70.4 |
| | 3.86, dd (*J*=10.5, 5.0) | - |
| 4 | 6.71, brs | 116.4 |
| 4a | - | 128.7 |
| 5 | - | 136.3 |
| 5a | 2.60, t (*J*=7.5) | 32.4 |
| 5b | 1.78, tt (*J*=7.5, 6.5) | 35.5 |
| 5c | 3.54, t (*J*=6.5) | 61.1 |
| 6 | 6.72, brs | 113.7 |
| 7 | - | 144.1 |
| 7a | - | 146.3 |
| 7 $OCH_3$ | 3.85, s | 55.4 |
| 1′ | - | 137.5 |
| 2′ | 7.03, d (*J*=2.0) | 110.8 |
| 3′ | - | 151.2 |
| 4′ | - | 145.0 |
| 5′ | 7.06, d (*J*=8.5) | 116.5 |
| 6′ | 6.89, dd (*J*=8.5, 2.0) | 118.1 |
| 3′ $OCH_3$ | 3.78, s | 55.5 |
| Rha 1′′ | 5.33, d (*J*=2.0) | 100.0 |
| 2′′ | 4.03, dd (*J*=1.75, 3.0) | 70.5 |
| 3′′ | 3.73, dd (*J*=5.5, 7.5) | 71.3 |
| 4′′ | 3.44, overlapped | 73.0 |
| 5′′ | 3.79, overlapped | 69.2 |
| 6′′ | 1.20 d (*J*=6.5) | 18.1 |

a) Measured in $CD_3OD$ (500 MHz for $^{1}H$ and 125 MHz for $^{13}C$)
*Assignment based on the HSQC and HMBC data.

## Antithrombotic activities of the compounds

Thrombogenesis (coagulation) is the process by which blood clots form, which is an integral part of hemostasis, wherein a damaged blood vessel wall is protected by a platelet and fibrin-containing clot to stop bleeding and begin repair of the damaged vessel. Disorders of thrombogenesis can result in an increased risk of hemorrhage or obstructive clotting (thrombosis) [4]. The effect of the purified compounds, the neolignan glycoside and icariside $E_4$, on thrombin inhibition was investigated by measuring thrombin clotting time in plasma at a final concentration of 500 μg/ml of the compounds. As a result, the clotting times of the neolignan. that the clotting of the neolignan glycoside was delayed four times compared to that of icariside $E_4$, suggesting that it was more effective for thrombin inhibition (Fig. 3A). In addition, an analysis of the inhibition effect by changing the concentration showed that the clotting time was delayed in accordance with an increase in the concentration of the neolignan glycoside, suggesting that it works in a concentration-dependent manner (Fig. 3B). Thrombin inhibition activity of the neolignan glycoside was approximately 417 sec at a final concentration of 1,000 μg/ml. This finding supports that the glycoside could be useful as an antithrombotic agent.

The secondary hemostasis coagulation cascade consists of two pathways leading to fibrin formation: the intrinsic coagulation pathway (also known as the contact activation pathway) and the extrinsic coagulation pathway (also known as the tissue factor pathway). These pathways are a series of reactions, in which a zymogen (inactive enzyme precursor) of a serine protease and its glycoprotein co-factor are activated, and catalyze the next reaction in the cascade, ultimately leading to cross-linked fibrin. The coagulation factors are generally serine proteases (enzymes) cleaving downstream proteins, whereas some of the enzymes are glycoproteins such as FVIII and FV, and the transglutaminase Factor XIII [2].

These coagulation factors circulate as inactive zymogens and activate the "final common pathway" of factor X, thrombin, and fibrin [9]. The intrinsic coagulation pathway begins with formation of the primary complex on collagen due to high-molecular-weight kininogen, prekallikrein, and FXII

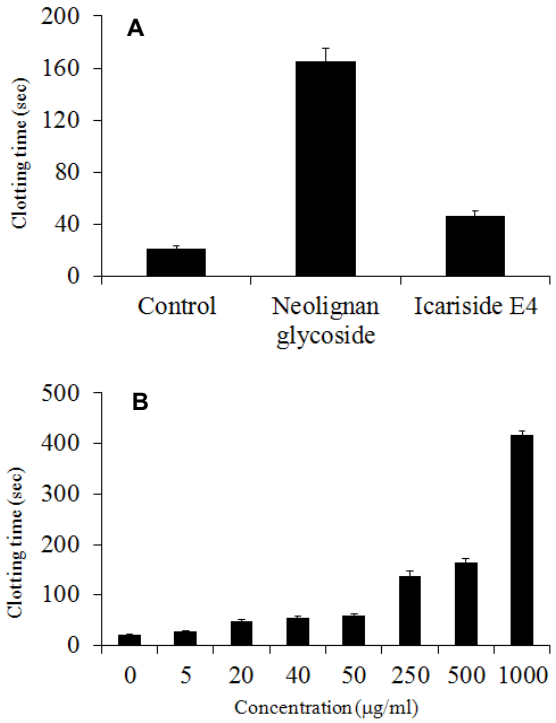


Fig. 3. Inhibition of thrombin by the purified neolignan glycoside and icariside E4 (A) and concentration dependent thrombin inhibition of neolignan glycoside (B). The compounds were mixed with thrombin, and pre-incubated for 2 min at 37°C. The clotting was determined by measuring the time consumed after adding and mixing plasma. Data are shown as mean ± SD.

(Hageman factor). Prekallikrein is converted to kallikrein and FXII becomes FXIIa, which converts FXI into FXIa. The resulting FXIa activates FIX, which forms the tenase complex with its co-factor FVIIIa, and FX is activated to FXa. In contrast, the main role of the extrinsic coagulation pathway is to generate a "thrombin burst", a process by which one of the most important coagulation constituents, thrombin, is released instantaneously. The primary function of thrombin is the conversion of fibrinogen to fibrin, the building block of the hemostasis process. The intrinsic and extrinsic coagulation pathways are activated and the coagulation cascade is maintained in a prothrombotic state by activation of FVIII and FIX to form the tenase complex.

Thrombin inhibitory factors occurring naturally in the human blood are generally glycoproteins containing a homologous amino acid composition. Based on the inhibition mode, antithrombin III inhibits the intrinsic blood clotting factors Xa, IXa, XIa, XIIa, and kallikerin, as well as thrombin activities, whereas the heparin cofactor II simply inhibits thrombin activity [6].

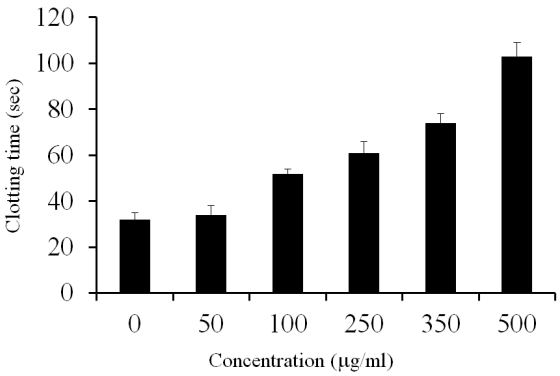


Fig. 4. Fibrinogen and thrombin binding inhibition of neolignan glycoside. The compounds were mixed with thrombin, and pre-incubated for 2 min at 37°C. The clotting was determined by measuring the time consumed after adding and mixing fibrinogen solution. Data are shown as mean ± SD.

The inhibitory mechanism of the isolated neolignan glycosides was investigated. The coagulation mechanism was examined by APTT (activited partial thromboplastin time) estimation method [23] confirming the inhibition of prothrombin blood coagulation factors Ⅴ, Ⅷ, Ⅸ, Ⅹ, Ⅺ, and Ⅻ activated in the plasma, and PT (prothrombin time) estimation method [22] confirming blood coagulation factors Ⅱ, Ⅴ,Ⅶ, Ⅹ and so on in plasma. As a result, the low level of blood coagulation activity similar to that of control group was significantly detected (data not shown). In addition, as shown in Fig. 4, analysis of TT (thrombin time) confirming the common pathway factor Ⅱ, Ⅴ, and fibrinogen suggested significantly the inhibition activity of blood coagulation. Thus, we examined the interaction of thrombin and fibrinogen to clarify the action mechanism (Fig. 4). As a result, the delay of coagulation time in the response of thrombin and pure fibrinogen may indicate that neolignan glycosides inhibit the thrombin action in a direct manner, leading to the suppression of fibrin generation. The inhibition activity was in accordance with the concentration-dependent manner. These findings suggest that neolignan glycosides, similarly to heparin, do not inhibit the thrombin response resulting from the activation of prothrombin inhibiting the factors in the plasma, but induce the blood coagulation delay by inhibiting the thrombin response of fibrinogen in the plasma. Further studies are needed on the inhibition mode of thrombin and fibrinogen interaction.

## Acknowledgement

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## 초록 : *Pinus densiflora* 유래의 항트롬빈 활성을 나타내는 Neolignan Glycoside의 동정

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소나무(*Pinus densiflora)*의 잎의 성분을 분리하여 항트롬빈 활성을 조사하였다. 잎은 70% ethanol로 3회 추출하였으며, 그 추출물은 순차적으로 chloroform과 *n*-buthanol로 분획하였다. *n*-buthanol 분획으로부터 얻은 수용성 분획을 MPLC와 HPLC에 의해 분리하였다. 분리한 compound를 $^{1}$H- 과 $^{13}$C-NMR로 동정한 결과 2, 3-dihydro - 7 - hydroxyl - 3 - hydroxymethyl - 2-(4´-hydroxyl - 3-methoxyphenyl) - 5 - benzofuranpropanol - 3 - *O* - α-rhamnopyranoside (a neolignan glycoside)와 2, 3-dihyro-3-hydroxymethyl-7-methoxy-2-(4´-hydroxyphenyl-3´- methoxy)-5-benxofuran propanol 4´-*O*-α-rhamnopyranoside (icariside $E_4$)의 neolignan 구조임을 확인하였다. 트롬빈 저해활성은 분리된 neolignan glycoside와 icariside $E_4$을 작용하여 혈장안에서 응고시간으로 측정하였다. 그 결과, neolignan glycoside의 응고시간이 icariside $E_4$보다 4배 이상 지연하였다. 저해활성은 neolignan glycoside의 농도가 증가함에 따라 그 시간이 지연되는 것을 확인하였다. 더 나아가 지연기전을 확인하기 위해 thrombin과 순수한 fibrinogen를 반응하였다. 그 결과, 트롬빈과 순수한 피브리노겐의 작용에 의해 응고시간은 지연되었으며, 이는 neolignan glycoside가 피브린형성에 주요한 트롬빈의 활성을 직접 저해하는 것으로 사료된다.