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Anti-Thrombosis Activity of Sinapic Acid Isolated from the Lees of Bokbunja Wine [S]

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From the lees of *bokbunja* wine (LBW) made from *Rubus coreanus* Miquel, we have identified six compounds (1: *trans*-4-hydroxycinnamic acid; 2: *trans*-4-hydroxy-3-methoxycinnamic acid; 3: 3,4-dihydroxycinnamic acid; 4: 4-hydroxy-3-methoxybenzoic acid; 5: 3,5-dimethoxy-4-hydroxybenzoic acid; and 6: 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid)) through silica gel chromatography and UHPLC-MS. The compounds 1−6 showed strong anti-coagulation and platelet aggregation inhibitory activities without hemolytic effect against human red blood cells. To date, this is the first report of the in vitro anti-thrombosis activity of sinapic acid. Our results suggest that different cinnamic and benzoic acid derivatives are closely linked to the anti-thrombosis activity of LBW, and sinapic acid could be developed as a promising anti-thrombosis agent.

Keywords: Anti-thrombosis activity, sinapic acid, lees of *bokbunja* wine, *Rubus coreanus* MIQ

Blood is essential to life, since it plays crucial roles in transferring oxygen, nutrients, and metabolic waste through the blood vessel, and regulating homeostasis of the body temperature, water content, buffer capacity, and immune system of the human body. Blood loss from damaged vessel is prevented by the formation of thrombus (blood clot), and the clots are degraded by a specific protease called plasmin [7]. Abnormally increased thrombus formation in the artery and vein system causes various thrombotic diseases, such as ischemic stroke, and myocardial infarction and is a major cause of morbidity and mortality [8]. Therefore, the precise and balanced regulation between thrombus formation and clot lysis is necessary.

Rubus coreanus Miquel (Rosaceae), the Korean black raspberry, has been traditionally used to treat diarrhea, stomach ailment, cancer, and paresthesia [7]. The immature fruit, called *bokbunja* in Korea, is mainly consumed as a fruit wine owing to its sour taste and low sugar content. The wine is famous in Southeast Asia for promotion of stamina and blood circulation. In spite of industrial production of this *bokbunja* wine (LBW), study of the lees of LBW is still rudimentary. The compressed LBW after wine production is discarded as it has no specific usage. Recently, we have reported that the ethylacetate fractions of the ethanol extract of LBW have strong anti-thrombosis activity via potent inhibition of blood coagulation and platelet aggregation [7]. However, the active compounds and its relevant mechanism have not yet been identified. Therefore, the aim of this study was to investigate and characterize novel anti-thrombotic compounds from LBW.

The compressed LBW from commercial facility was provided by Kuksoondang Co. (South Korea). The pH, brix, and water content of the LBW were 3.96, 2.0, and 50%, respectively. A 20 kg of amount LBW was extracted three times with 200 L of ethanol (95%; Deajong, Korea) and the combined extract was successively partitioned with *n*-hexane, ethylacetate, and butanol. The organic solvent fractions and the water residue were concentrated by a rotary vacuum evaporator (Eyela Co., N-1110VW, Japan). The ethylacetate fraction was further purified using silica-
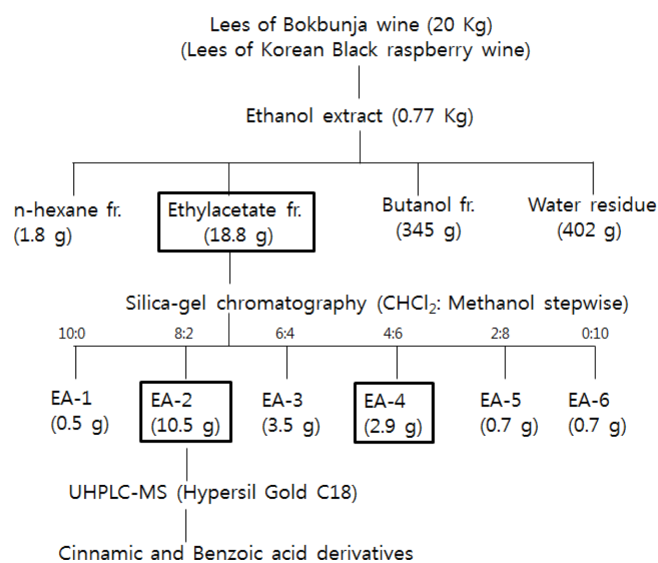
Fig. 1. Methodology used for identification of cinnamic and benzoic acid derivatives in lees of *bokbunja* wine.
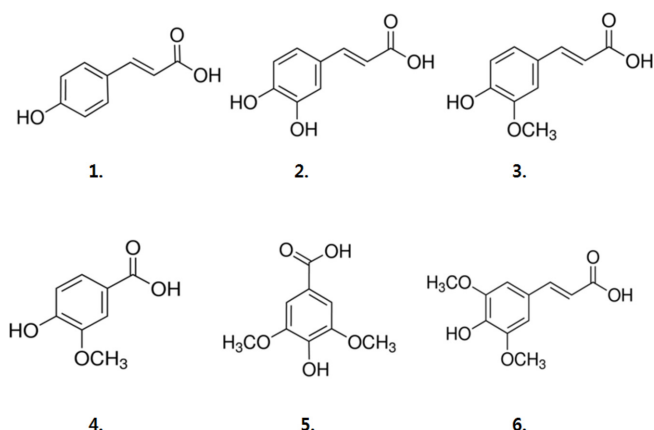

Fig. 2. Structure of the identified cinnamic acid and benzoic acid derivatives isolated from the lees of *bokbunja* wine.

1: *trans*-4-hydroxycinnamic acid (*p*-coumaric acid); 2: *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic acid); 3: 3,4-dihydroxycinnamic acid (caffeic acid); 4: 4-hydroxy-3-methoxybenzoic acid (vanillic acid); 5: 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid); and 6: 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid).

gel chromatography (Duran, Germany) in which various mixtures of methylene chloride:methanol (10:0, 8:2, 6:4, 4:6, 2:8, and 0:10 (v/v)) were eluted as a mobile phase in a stepwise manner (Fig. 1). Each fraction was subjected to TLC, using a silica gel plate (silica gel $60F_{254}$; Merck, Darmstadt, Germany) with running solvent of chloroform: methanol:water (52:28:8 (v/v/v), lower layer). The EA-2 fraction was subjected to UHPLC-MS to determine the active compounds of LBW. A Thermo Scientific system (Ultimate 3000; Thermo Fisher Scientific Inc., USA) consisting of binary solvent manager and sample manager was coupled with a Thermo Scientific Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific Inc.). All UHPLC analyses were performed on the analytical column Hypersil Gold C18 (50 mm × 2.1 mm, 1.9 μm; Thermo Fisher Scientific Inc.) and the column was maintained at 30ºC. Samples were separated using a gradient elution with 0.1% formic acid and 5 mM ammonium acetate in water (solvent A) and methanol (solvent B). The flow rate was set at 0.25 ml/min and the chromatographic run time was 10.0 min, including equilibration of the system. The gradient was started with 2% of solvent B, was increased to 95.0% over 1 min, and in 8.0 min the percentage of solvent B was ramped to 2%. The injection volume was 5 μl. The ion source (heated electrospray ionization) and ion optic parameters were optimized. By UHPLC-MS analysis, six compounds were detected from the EA-2 fraction and were identified as (**1**) *trans*-4-hydroxycinnamic acid (*p*-coumaric acid), (**2**) *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic

acid), (**3**) 3,4-dihydroxycinnamic acid (caffeic acid), (**4**) 4-hydroxy-3-methoxybenzoic acid (vanillic acid), (**5**) 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), and (**6**) 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid), respectively (Fig. 2). Among them, compound **6** has not been reported in *R. coreanus* Miquel, and it might be produced during wine fermentation. The calculated content for the identified compounds (**1**–**6**) were 0.02, 0.18, 0.75, 145.8, 134.7, and 10.78 mg/100 g-LBW, respectively.

Sinapic acid is found in fruits, vegetables, cereal grains, and oilseed crops [10, 13]. It shows various biological activities, such as antioxidant [3, 4], antibacterial [16], chemo-preventive anticancer [1], anti-inflammatory [19, 20], anti-hyperglycemic [2], and peroxynitrite scavenging activities [21]. It is also famous for anxiolytic [18], neuro-protective [5, 7], and cognition-improving activities [5, 15] and protective effect on neural damage against toxic chemicals [6, 9, 11, 12]. Although the protective effect of sinapic acid against heart ischemia/reperfusion injury is recently reported [17], the anti-coagulation and platelet aggregation inhibitory activities of sinapic acid are not reported.

Thrombin is the key enzyme of hemostasis, which includes the conversion of fibrinogen to fibrin network, platelet aggregation, and feedback amplification of coagulation. The activation of thrombin at sites of vascular injury needs an ordered series of activation of prothrombin and blood coagulation factors [8]. Therefore, compounds **1**–**6** were

Table 1. Anti-coagulation activities of the identified compounds from the lees of *bokbunja* wine.

Compounds	Conc. (mg/ml)	Anti-coagulation activity[1]		
		TT[2]	PT[3]	aPTT[4]
DMSO	-	1.00 ± 0.06^a	1.00 ± 0.02^a	1.00 ± 0.05^a
Aspirin	5.0	$>15^f$	$>15^e$	$>15^g$
	2.5	7.74 ± 0.50^e	$>15^e$	4.21 ± 0.08^e
	1.5	1.87 ± 0.21^b	1.35 ± 0.07^a	1.71 ± 0.16^b
p-Coumaric acid	5.0	$>15^f$	$>15^e$	$>15^g$
	2.5	$>15^f$	$>15^e$	5.30 ± 0.26^f
	1.3	1.32 ± 0.05^a	1.27 ± 0.13^a	0.92 ± 0.01^a
Ferulic acid	5.0	$>15^f$	$>15^e$	$>15^g$
	2.5	1.97 ± 0.31^b	3.05 ± 0.29^c	1.67 ± 0.33^b
	1.3	1.21 ± 0.15^a	1.21 ± 0.00^a	1.15 ± 0.13^a
Caffeic acid	5.0	$>15^f$	$>15^e$	$>15^g$
	2.5	3.00 ± 0.12^d	4.51 ± 0.79^d	2.76 ± 0.30^d
	1.3	1.05 ± 0.00^a	1.28 ± 0.03^a	1.07 ± 0.08^a
Vanillic acid	5.0	$>15^f$	$>15^e$	$>15^g$
	2.5	3.25 ± 0.51^d	$>15^e$	2.06 ± 0.03^c
	1.3	1.27 ± 0.16^a	1.21 ± 0.01^a	1.01 ± 0.05^a
Syringic acid	5.0	$>15^f$	$>15^e$	$>15^g$
	2.5	2.57 ± 0.16^c	2.30 ± 0.14^b	2.05 ± 0.00^c
	1.3	1.15 ± 0.07^a	1.27 ± 0.11^a	1.07 ± 0.10^a
Sinapic acid	5.0	$>15^f$	$>15^e$	$>15^g$
	2.5	2.17 ± 0.38^b	2.34 ± 0.14^b	1.12 ± 0.03^a
	1.3	1.21 ± 0.12^a	1.24 ± 0.08^a	1.07 ± 0.05^a

[1]Anti-coagulation activity: the coagulation time by addition of pure compound divided by the coagulation time by addition of DMSO as solvent control.

[2]TT: Thrombin time; [3]PT: Prothrombin time; and [4]aPTT: activated partial thromboplastin time.

The TT, PT, and aPTT of solvent control (dimethylsulfoxide) were 24.0 sec, 18.6 sec, and 40.5 sec, respectively. Different letters within a column differ significantly ($p < 0.05$).

dissolved in dimethylsulfoxide, and the anti-coagulation activity was measured by determining the clotting time required for activation of thrombin (TT: thrombin time), prothrombin (PT: prothrombin time), and coagulation factors (aPTT: activated partial thromboplastin time), respectively. All data are presented as the mean ± SD values of triplicates. The TT, PT, and aPTT of compounds **1–6** and aspirin (5 mg/ml) were prolonged to 15-folds compared with those of control (Table 1). In addition, coumaric acid extended the TT and PT to 15-folds at 2.5 mg/ml, and vanillic acid and aspirin extended the PT to 15-folds at 2.5 mg/ml. These results suggest that different cinnamic and benzoic acids are related to the anti-coagulation activity of LBW, and sinapic acid has potent anti-coagulation activity.

The anti-thrombosis activities are dependent on platelet the aggregation inhibitory activity and/or anti-coagulation activity. Therefore, the effects of compounds **1–6** on platelet aggregation were measured using the Whole Blood Aggregometer (Chrono-log, PA, USA). The PRP obtained from human blood was washed with washing buffer, and the washed platelets (5×10^8 cells/ml) were suspended using buffer containing 138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, 0.49 mM $MgCl_2$, and 0.25% gelatin (pH 7.4). Then the platelets were incubated with compounds **1–6** and followed by stimulation with 2.5 ml of collagen (1 mg/ml) at 37C for 12 min. Addition of the compounds **1–6** into the suspension buffer did not show any pH changes. During the aggregation, the amplitude (expressed ohms by maximum extent of platelet aggregation), slope (rate of reaction determined by drawing a tangent through the steepest part of the curve), and area under (a calculated area in descent drawing

Table 2. Effects of the identified compounds from the lees of *bokbunja* wine on platelet aggregation.

Compounds (mg/ml)	Conc. (mM)	Amplitude (Ω)	Slope (Ω/min)	Lag time (sec)	Area under	PAA[1] (%)
DMSO	-	17	4	29	119.7	100.0
Aspirin (0.50)	2.78	9	2	130	45.5	38.0
Aspirin (0.25)	1.39	13	2	30	87.2	72.9
p-Coumaric acid (0.25)	1.52	6	1	84	36.0	30.1
p-Coumaric acid (0.20)	1.22	10	1	38	63.9	53.4
p-Coumaric acid (0.15)	0.91	16	2	25	118.9	85.7
Ferulic acid (0.25)	1.29	13	2	1	86.6	72.4
Ferulic acid (0.20)	1.03	17	2	28	105.8	88.4
Caffeic acid (0.25)	1.39	10	2	36	64.5	53.9
Caffeic acid (0.20)	1.11	17	2	30	103.1	86.2
Vanillic acid (0.25)	1.49	13	2	33	81.2	67.9
Syringic acid (0.25)	1.26	13	2	30	84.1	70.3
Sinapic acid (0.25)	1.12	6	1	138	35.3	29.5
Sinapic acid (0.20)	0.89	14	2	32	102.7	85.8

[1]PAA: platelet aggregation activity.

during platelet aggregation) were determined [7]. As shown in Table 2, aspirin showed potent aggregation inhibition in a concentration-dependent manner. The IC_{50} of aspirin was calculated to 0.412 mg/ml (2.29 mM). Addition of the compounds **1−6** into platelets also inhibited the aggregation in a concentration-dependent manners although the inhibitions of each compound were different. Among the compounds, coumaric acid and sinapic acid showed potent aggregation inhibitory activity. The platelet aggregations were decreased to 30% by addition of coumaric acid and sinapic acid (0.25 mg/ml), which were comparable to that of aspirin. The IC_{50} values of coumaric acid and sinapic acid were calculated to 0.212 mg/ml (1.29 mM) and 0.232 mg/ml (1.03 mM), respectively (Fig. S1). To date, there has been no previous report of the inhibitory activity of sinapic acid against blood coagulation and platelet aggregation, to our knowledge. The hemolytic activity of compounds **1−6** was evaluated by determining the release of hemoglobin from a 4% suspension of fresh erythrocytes at 414 nm with an ELISA plate reader [14]. Hemolytic ratios of 0% and 100% were determined in DMSO (2%)-containing PBS and 0.1% Triton X-100, respectively. The hemolytic activities of the compounds were negligible up to 0.5 mg/ml concentration. These results suggest that the LBW has high potential as a novel resource of functional food, and sinapic acid could be developed as a promising anti-thrombosis agent.

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