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Antithrombotic Compounds from the Leaves of *Ligularia stenocephala* M.

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Abstract – The leaf extract of *Ligularia stenocephala* showed the highest anti-platelet aggregating activity in large numbers of edible and herbal plants. The active fraction fractionationed from *L. stenocephala* extract by using preparative HPLC inhibited the platelet aggregation up to above 80% and its blood coagulating time (PT and APTT) also showed similar effect to aspirin (0.2 μg/mL), known as an anti-thrombus compound. Two antithrombus active compounds were purified and identified as 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid, respectively, on the basis of NMR and FAB-MS spectroscopic data. Two active compounds has not only antiplatelet aggregating activity, but also has anticoagulating activity.
Keywords – *Ligularia stenocephala* M., 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, antithrombus activity

Introduction

Platelet aggregation is a crucial factor in the pathogenesis of ischemic disease (Hus *et al.*, 1986). Because they readily aggregate in response to a variety of endogenous substances and secrete various substances that cause further aggregation, platelets can initiate thrombus formation and precipitate thromboembolism leading to ischemic diseases. In addition, substances secreted from platelets can mediate many other biologic reactions and may also be involved in atherogenesis and other pathologic processes. Hence, drugs that inhibit platelet aggregation or secretion could have wide biological implications. However, the present state of knowledge has not approached the point where one can rationally design new classes of molecules which could be expected to be effective. Plants have been considered especially valuable in the empirical search for new drugs in such situations because plants contain broad classes of compounds as their constituents (Yun-Choi *et al.*, 1995). Indeed, a number of valuable drugs are either directly derived from plants or synthesized using plant components as prototypes (Hara *et al.*, 1991; Paik *et al.*, 1995). In addition, there is a voluminous history of the use of plants in folklore for the treatment of symptoms related to thromboembolic disorders.

We have previously reported a research on screening of platelete aggregating inhibitors from 130 varieties of edible and herbal plants (Yoon *et al.*, 1997). During secondary screening for thrombus inhibitors from these selected plants, *L. stenocephala* appeared to have the highest antithrombus activity. The genus *Ligularia* belong to the family *Compositae* has been widely distributed in eastern and southern Asia, and has been used for the treatment of influenza, cough, ulcer and tuberculosis in the traditional medicine (Choi *et al.*, 2004). The authors have isolated two phenylpropanoid compounds from the EtOAc-soluble fraction of the leaves of *L. stenocephala* and tested the antithrombus activity. This paper describes the structural elucidation of two active compounds (**1** - **2**) and their biological activity.

Experimental

General – High performance liquid chromatography (HPLC) were performed on Jasco LC-900 apparatus (Jasco, Tokyo, Japan) equipped with L-4200H UV/Vis detector using a Develsil octadecyl silica (ODS) column (20 × 250 mm). Optical rotations were measured on a JASCO model J-5 in MeOH solution at 25 °C. The IR spectra were determined on a Pye Unicam SP 3200 Infra-Red Spectrophotometer (Philips, Netherland). Mass spectra were measured by using a Jeol JMS-BX 303 spectrometer in the FAB mode using glycerol matrix with polyethylene glycol as internal standard. The ^{1}H- and ^{13}C-NMR spectra were determined on a Varian UNITY 300 (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR) in CD_3OD, using

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the tetramethylsilane (TMS) signal as an internal standard. Analytical silica gel TLC (Kiesel gel 60 F254, 0.25 mm, Merck, Germany) plates were used without activation. Antiaggregating activity was measured by using Chron-Log Aggrometer, Aggro/Link 810A.

Plants – The leaf of L. stenocephala were collected at Pyungchang, Kangwon Province, Korea. Fresh leaves were dried in a dark, well-ventilated place.

Extraction and isolation – The dried leaves of L. stenocephala (4.0 kg) were ground into powder and extracted with 80% aqueous MeOH twice at room temperature for 4 days. The MeOH extract was filtered and concentrated under reduced pressure. The extract was partitioned successively between H_2O and $CHCl_3$, EtOAc (45.2 g) and then BuOH (92.8 g). During the isolation procedure, the antithrombus activity was monitored by the assay using modified thin smear method. The EtOAc-soluble fraction was chromatographed on silica gel column (2 kg, 6 × 100 cm, toluene/EtOAc/MeOH/formic acid = 5 : 3.5 : 0.5 : 1, v/v) and followed -gel column (acetone/H_2O = 1 : 9 at beginning and 6 : 4 at the end, v/v). The active compounds were finally purified by reverse-phase HPLC (Develosil ODS-10/20, ODS, 20 × 250 mm, aq. 53% MeOH: 0.1% formic acid) yielded compound **1** (1.5 g) and compound **2** (0.9 g).

Compound 1 – Amorphous powder; $[\alpha]_D^{25}$ −69.6° (*c* 0.9, MeOH) ; IR (KBr) cm^{-1}: 3200, 1700, 1620, 1530, 1460, 1380, 1290, 1195, 1130, 995, 870, 825; FAB-MS *m/z*: 539 $[M+Na]^+$, 517 $[M+H]^+$; UV λ_{max} nm: 336, 300, 225; ^1H- and ^{13}C-NMR data: see Table 3.

Compound 2 – Amorphous powder; $[\alpha]_D^{25}$ −120.5° (*c* 1.2, MeOH); IR (KBr) cm^{-1}: 3210, 1715, 1620, 1520, 1360, 1310, 1190, 1125; FAB-MS *m/z*: 539 $[M+Na]^+$, 517 $[M+H]^+$; ^1H- and ^{13}C-NMR data: see Table 3.

Preparation of platelet rich plasma (PRP) - A male Sprague-Dawley rat (220 ± 30 g) was anesthetized with $CHCl_3$ and blood drawn from the heart into a plastic syringe containing 1/10 volume of 2.2% trisodium citrate. The citrated blood was centrifuged at 200 × g for 10 min at room temperature, and supernatant platelet rich plasma (PRP) was obtained.

Estimation of antiplatelete aggregating activity (modified smear method) – Modified thin smear method was performed for measuring antiplatelete aggregating activity (Yun-Choi *et al.,* 1985). The test samples were dissolved in saline, while H_2O-insoluble samples were first dissolved in EtOH and then diluted in saline to give 1% of the final EtOH concentration. The 20 μL of sample solution was added to 160 μL of PRP and 2 min after preincubation at 37 °C for collagen (2 μg/mL) or at room temperature for adenosine 5'-diphosphate (ADP, 1 μg/mL) as the aggregating agents, 20 μL of the appropriate aggregating agent was added, and the tube was vigorously agitated for 10 sec. Thin smears were prepared on glass slides after incubation for 4 min and fixed in EtOH and stained with a Wright-Giemsa stain. The smears were subjected to examination under an ordinary light microscope using an oil immersion objective lens (1000 times). Aspirin and ADP was used as positive control and negative control, respectively, to insure control smears.

Estimation of antiplatelete aggregating activity (electrical impedence method) – Electrical impedence method was employed as other method for estimation of antiaggregating activity. The test sample solution (0.05 mL) was mixed with whole blood (0.45 mL) containing 1/10 volume of 2.2% trisodium citrate and saline (0.45 mL) and 0.05 mL of aggregating agents, ADP and collagen were added into the mixture. antiaggregating activity of the sample was determined with Chron-Log Aggrometer, Aggro/Link 810A (USA) by measuring the impedence values, which is the degree of aggregation of platelets induced between two electrode for 8 min at 37 °C.

Antiblood coagulating activity – Analysis of prothrombin time (PT) and activated prothrombin time (APTT) was conducted with PRP of rat using Blood Coagulator (ACL-100, Instrumentration Lab. USA) to estimate anti-blood coagulating activity (Tr) for the active fraction of *L. stenocephala.*

Results and Discussion

The antiplatelet aggregating activity was estimated with ADP or collagen as the aggregating agent by using modified thin smear method and electrical impedence method to select potential inhibitors of platelet aggregation from large numbers of edible plant extracts screened in previous report (Yoon *et al.,* 1997). Among plant screened, the extract of *L. stenocephala*, *Aster scaber* and *Allium victorialis* showed high anti-aggregating activities against ADP and collagen, and the apparent result under two assay methods were coincided well (Table 1). The *L. stenocephala* extract showed the highest activity was employed further to isolate an anti-platelet aggregating compound.

The effects of *L. stenocephala* on anti-platelet aggregating activity were examined with the active fractions (Peak-H1 and Peak-H2) of the extract separated from preparative HPLC (data not shown). The platelet aggregation was inhibited up to above 80% by peak-H1 fraction and its blood coagulating time (PT and APTT)

Table 1. Antiplatelet aggregating activities of edible and herbal plants

Scientific name	Tissue[b]	Thin smear method[a]		Impedence method	
		ADP (1 μg/mL)	Collagen (2 μg/mL)	ADP (2 μg/mL)	Collagen (3 μg/mL)
Compositaceae					
Aster scaber	L	–	–	0	5
Ligularia stenocephala	L	–	–	0	0
Taraxacum mongolicum	L	–	±	5	15
Campanulaceae					
Platycodon glaucum	R	–	–	10	20
Apiaceae					
Angelica gigas Nakai	R	–	–	5	15
Liliaceae					
Allium victorialis L.	C	–	–	5	5
Moraceae					
Morus bombycis Koidzumi	R	–	–	10	20
Portulacaceae					
Portulaca oleracea L.	Wp	–	–	+	
Araliaceae					
Acanthopanax sessiliflorus	Co	–	–	5	15
Boraginaceae					
Lappla deflexa Garck	R	–	±	5	25
Tiliaceae					
Alisma plantago	R	–	±	10	20
Graminales					
Phyllostachys reticulata	L	–	±	10	20

[a]Anti platelet-aggregation activities: –, no aggregation; ±, slight aggregation; +, intermediate aggregation;, ++ and +++, full aggregation.
[b]Tissues of the plant body used for extraction are indicated as follows: At, aerial tuber; Co, cortex; Fr, fruit; L, leaf; R, root; St, stem; and Wp, whole plant.

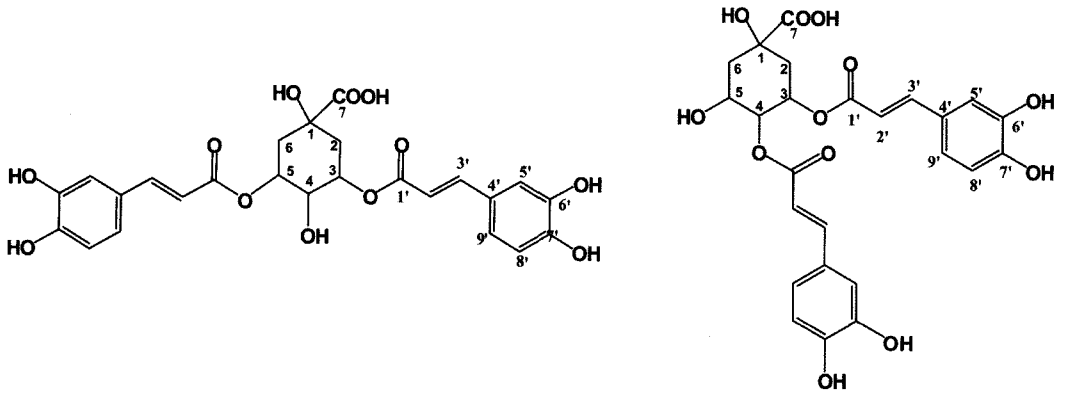
Table 2. Antithrombus activity of the active fractions separated by HPLC from *Ligularia stenocephala*

Sample	Platelete aggregating inhibition (%)[a]		Coagulating activity (sec)[b]	
	ADP (1 μg/mL)	Collagen (2 μg/mL)	PT	APTT
Control[c]	94	91	14.2	25.4
Peak-H1	82	84	13.8	24.9
Peak-H2	15	19	12.9	23.8

[a]Platelete aggregating inhibition by electrical impedence method.
[b]Blood coagulating retention time (Tr) estimated with PT and APTT.
[c]Aspirin (2×10^{-1} μg/mL) known as anti-thrombus compound.

also showed similar effect to aspirin (0.2 μg/mL) known well as anti-thrombus compound at the concentration of 5 μg/mL (Table 2). These results proved that the compound in active fraction (Peak-H1) must be an anti-thrombus component, because an anti-platelet aggregating compound inhibit not only the production of Thromboxane A2 (TAX_2), an platelet aggregation promoting factor, but also promote the generation of cAMP and cGMP act as inhibitor of TAX_2 (Hara *et al.*, 1991). The active fractions (Peak-H1 and Peak-H2) was further used for identifying the structure of the compounds.

The molecular formula of compound **1** was established as $C_{25}H_{24}O_{12}$ by FAB-MS spectroscopy (*m/z* 539 $[M+Na]^+$ and 517 $[M+H]^+$) in combination with ^1H- and ^{13}C-NMR spectra data. The IR spectrum suggested the presence of hydroxyl (3200 cm^{-1}) and α,β-unsaturated



1 2

Fig. 1. Structure of compounds **1-2** from *Ligularia stenocephala.*

Table 3. NMR (^{1}H, 300 MHz; ^{13}C, 75 MHz) data for compounds **1 - 2** in CD_3OD

Position	Compound **1**		Compound **2**	
	δH (*J*, in Hz)	δC	δH (*J*, in Hz)	δC
1		74.2		75.6
2	2.11 (1H, m)	37.6	2.09 (1H, m)	39.4
	2.33 (1H, m)		2.51 (1H, m)	
3	5.43 (1H, m)	72.1	5.64 (1H, m)	67.1
4	3.99 (1H, dd, 7.2, 2.7)	70.6	5.11 (1H, m)	74.9
5	5.39 (1H, m)	72.5	4.40 (1H, m)	69.1
6	2.23 (1H, m)	35.9	2.16 (1H, m)	37.3
	2.31 (1H, m)		2.34 (1H, m)	
7		177.8		174.4
1'		166.8		166.4
		168.3		168.3
2'	6.27 (1H, d, 15.9)	115.1	6.18 (1H, d, 15.9)	115.2
	6.35 (1H, d, 15.9)	115.2	6.27 (1H, d, 15.9)	115.2
3'	7.57 (1H, d, 15.9)	147.3	7.50 (1H, d, 15.9)	145.5
	7.63 (1H, d, 15.9)		7.58 (1H, d, 15.9)	
4'		123.0		127.2
5'	7.06 (2H, d, 1.8)	115.1	6.99 (2H, s)	109.7
6'		146.7		149.2
7'		149.6		151.3
8'	6.78 (2H, d, 8.1)	115.6	6.74 (2H, d, 7.2)	111.1
		116.5		110.5
9'	6.97 (2H, dd, 8.1, 1.9)	122.9	6.89 (2H, d, 7.2)	122.9
		123.0		123.0

carbonyl (1700 cm^{-1}) groups. The ^{1}H-NMR spectrum of compound **1** showed signals for six aromatic methines at δ 7.06, 6.97 and 6.78, and four *trans* conjugated olefinic groups (J = 15.9 Hz) at δ 6.27, 6.35, 7.57 and 7.63 (Table 1). These data were identified as caffeic acid. The presence of caffeic acid was also supported by the UV maxima at 336, 300 and 225 nm. Additional signals in the ^{1}H-NMR spectrum were typical for a quinic acid, such as methine protons at 5.43, 3.99 and 5.39. These data indicated that the structure of compound **1** was composed of two caffeic acids and a quinic acid. The two carbon positions of quinic acid moiety combined to caffeoyl acids were identified by comparing chemical shifts of free quinic acid proton. Compared to free quinic acid, the protons of at C-3 and C-5 of compound **1** were shifted downfield by 1.4 ppm. Furthermore, the ^{13}C-NMR chemical shifts of all signals due to the quinic acid of compound **1** were in good agreement with those of quinic acid, except for C-3 and C-5. Therfore, the two caffeic acid moieties are combined to the hydroxyl groups at C-3 and C-5. Conclusively, compound **1** was assigned as 3,5-dicaffeoylquinic acid (Fig. 1).

Compound **2** was closely related to compound **1** in its physicochemical properties and NMR spectra, suggesting that compound **2** has a quinic acid and two caffeic acid moieties. The molecular formula was determined to be $C_{25}H_{24}O_{12}$ by FAB mass spectroscopy (*m/z*, 539 $[M+Na]^+$ and 517 $[M+H]^+$) in combination with ^{1}H- and ^{13}C-NMR spectra data. However, compound **2** differed from compound **1** in the ^{1}H- and ^{13}C-NMR chemical shifts at H-4, H-5, C-3, C-4, and C-5. The signals of H-5, C-5 in **2** were shifted upfield from those of **1**, while H-4, C-3, and C-4 were shifted downfield. From the above results, the structure of **2** was assigned as 3,4-dicaffeoylquinic acid (Fig. 1).

The active fraction isolated from *L. stenocephala* inhibited the platelet aggregation up to above 80% and its blood coagulating time (PT and APTT) also showed similar effect to aspirin (0.2 μg/mL) known well as anti-thrombus agent at the concentration of 5 μg/mL. The apparent result is to prove that the active compound has not only an antiplatelet aggregating activity, but also has an anticoagulating activity.

Two antithrombus active compounds **1** - **2** were identified as 3,5-dicaffeonylquinic acid and 3,4-dicaffeonylquinic acid, respectively. The quinic acid and/or caffeic acid have been reported to have a variety of biological activities as the following effects, such as inhibition against tyrosinase (Iwai *et al.*, 2004; Hur *et al.*, 2004), aldose reductase (Terashima *et al.*, 1991), chemiluminescence (Heilmann *et al.*, 1995) and inhibition against production of serum triglyceride and total cholesterol (Iwai *et al.*, 2004), as well as lipid peroxide (Makoto *et al.*, 1998; Nishizawa *et al.*, 1998), antioxidant (Kim *et al.*, 2007; Lee *et al.*, 2004; Wang *et al.*, 2003) and neuroprotective effects (Kim *et al.*, 2005). Although *L. stenocephala* has been known as the treatment of influenza, cough, ulcer, and tuberculosis in the traditional medicine (Choi *et al.*, 2004), it is interesting that there was not almost the report on this plant associated with an antithrombus or antiplatelet activiy. Moreover, the presence of 3,4-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid in *L. stenocephala* is reported first by this work.

References

Choi, J.W., Park, K., Lee, K.T., Park, K.K., Kim, W.B., Lee, J.H., Jung, H.J., and Park, H.J., Inhibitory effect of *Ligularia fischeri* var. *spiciformis* and its active component, 3,4-dicaffeoylquinic acid on the hepatic lipid peroxidation in acetaminophen-treated rat. *Natural Product Sciences* **10**, 182-189 (2004).

Hara, H., Kitajima, A., Shimada, H., and Tamao, Y., Antithrombotic effect of MCI-9024, a new antiplatelet agent experimental thrombosis models. *Thromb. Haemorr.* **66**, 484 (1991).

Heilmann, J., Merfort, I., and Weiss, M., Radical scavenger activity of different 3',4'-dihydroxyflavonols and 1,5-dicaffeoylquinic acid studied by inhibition of chemiluminescence. *Planta Medica* **61**, 435-438 (1995).

Hsu, H., Chen, Y.P., Shen, S.J., Hsu, C.S., Chen, C.C., and Chang, H.C., *Oriental Materia Medica, a Concise Guide*, pp 503-04. Oriental Healing Arts Institute, Taiwan, 1986.

Hur, J.Y., Lee, P.J., Kim, H.C., Kang, I.S., Lee, K.R., and Kim, S,Y., (−)-3,5-Dicaffeoyl-muco-quinic acid isolated from *Aster scaber* contributes to the differentiation of PC12 cells: through tyrosine kinase cascade signaling. *Biochemical and biophysical research communications* **313**, 948-953 (2004).

Iwai, K., Kishimoto, N., Kakino, Y., Mochida, K., and Fujita, T., In vitro antioxidative effects and tyrosinase inhibitory activities of seven hydroxycinnamoyl derivatives in green coffee beans. *Journal of Agricultural and Food Chemistry* **52**, 4893-4898 (2004).

Kim, C.M., Kwon, Y.S., and Yun-Choi, H.S., Antithrombotic effect of BuOH soluble fraction of *Angelica dahurica* root. *Kor. J. Pharmacogn.* **26**, 74-77 (1995).

Kim, H.J., Jin, C.B., and Lee, Y.S., Isolation and antioxidative activities of caffeoylquinic acid derivatives and flavonoid glycosides from leaves of sweet potato (*Ipomoea batatas* L.). *Journal of Applied Pharmacology* **15**, 46-51 (2007).

Kim, S.S., Park, R.Y., Jeon, H.J., Kwon, Y.S., and Chun, W.J., Neuroprotective effects of 3,5-dicaffeoylquinic acid on hydrogen peroxide-induced cell death in SH-SY5Y cells. *Phytotherapy Research* **19**, 243-245 (2005).

Lee, S.E., Chung, T.Y., Seong, N.S., and Bang, J.K., In vitro antioxidant activity of 3,5- dicaffeoylquinic acid isolated from *Aster scaber* Thumb. *Agricultural Chemistry and Biotechnology* **47**, 27-30 (2004).

Makoto, N., Ruriko, I., Ko, K., Yasuko, K., and Yasuo, F., 5-lipoxy inhibitors from Gardeniae fructus. *Chem. Pharm. Bull.* **36**, 87-95 (1988).

Nishizawa, M., Izuhara, P., Kaneko, K., Koshihara, Y., and Fujimoto, Y. 5-Lipoxygenase inhibitors isolated from *Gardeniae fructus*. *Chem.*

Pharm. Bull. **35**, 87-95 (1988).

Paik, Y.S., Song, C.H., Chung, K.S., and Yun-Choi, H.S., Anti-platelet and anti-thrombotic effects of *Gastrodia elata*. *Kor. J. Pharmacogn.* **26(4)**, 385-389 (1995).

Robinson, W.E., Cordeiro, M, Abdel-Malek, S., Jia, Q., Chow, S.A., Reinecke, M.G., and Mitchell, W.M, Dicaffeoylquinic acid inhibitors of human immunodeficiency virus integrase: inhibition of the core catalytic domain of human immunodeficiency virus integrase. *Molecular pharmacology* **50**, 846-855 (1996).

Terashima, S., Shimizu, M., Horie, S., and Morita, N., Studies on aldose reductase inhibitors from natural products. IV. Constituents and aldose reductase inhibitory effect of *Chrysanthemum morifolium*, *Bixa orellana* and *Ipomoea batatas*. *Chem. Pharm. Bull.* **39**, 3346-3347 (1991).

Wang, S.Y., Chang, H.N., Lin, K.T., Lo, C.P., Yang, N.S., and Shyur, L.F., Antioxidant properties and phytochemical characteristics of extracts from *Lactuca indica*. *Journal of Agricultural and Food Chemistry* **51**, 1506-1512 (2003).

Yun-Choi, H.S., Chung, K.S., Lim, M.H., and Oh, J.H., Antithrombotic effects of some traditional plant medicines. *Kor. J. Pharmacogn.* **26**, 154-158 (1995).

Yun-Choi, H.S., Kim, S.O., Kim, J.H., Lee, J.R., and Cho, H.I., Modified smear method for screening potential inhibitors of platelet aggregation from plant sources, *J. Nat. Prod.* **48**, 363 (1985).

Yoon, M.H., Lim, C.H., Oh, J.H., Lee, J.C., and Choi, W.Y., Screening of Inhibitors of platelet aggregation from edible plants. *Jour. Agri. Sci. Chungnam Nat'l Univ.* **24**, 267-274 (1997).

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