Antioxidant Activities of Acetylmartynosides from *Clerodendron trichotomum*

Sungwook Chae[1,3], Kyoung Ah Kang[2], Ju Sun Kim[1], Ho Kyoung Kim[3], Eun Ju Lee[1], Jin Won Hyun[2,*] and Sam Sik Kang[1,*]

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

[2]*Department of Biochemistry, College of Medicine and Applied Radiological Science Research Institute, Cheju National University, Jeju-si 690-756, Korea*

[3]*Department of Herbal Resources Research, Korea Institute of Oriental Medicine, Daejeon 305-811, Korea*

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Two acetylmartynosides, 2''-acetylmartynoside and 3''-acetylmartynoside were isolated from the CH_2Cl_2 fraction of *Clerodendron trichotomum* (Verbenaceae) by open column chromatography and MPLC. Their chemical structures were identified by the direct comparison of spectral data reported in the literature, and their antioxidant properties were evaluated. Both compounds showed antioxidant activity based on evaluations of intracellular ROS and DPPH radical scavenging activities.

Key words: *2''-acetylmartynoside, 3''-acetylmartynoside, antioxidant activity,* Clerodendron trichotomum, *intracellular ROS scavenging activity*

Polyphenols refer to one of the numerous and widely distributed chemical groups in the plant kingdom. Based on their structures they are classified into different families, phenolic acids, phenylpropanoids, flavonoids, or the less common lignans and stilbenes. Polyphenols are attracting interest because of their numerous biological effects such as antioxidant, anti-aging, anti-inflammation, anti-hypertension, and anticancer effects [Bravo, 1998]. Moreover, the polyphenols may contribute to the prevention of a wide range of common diseases by scavenging ROS, which include free radicals, such as the superoxide anion (O_2^-) and the hydroxy radical (OH·), and non radical species like hydrogen peroxide (H_2O_2) [Halliwell and Gutteridge, 1998]. Superoxide dismutase (SOD) defends biological systems from potentially harmful ROS levels by catalyzing superoxide anion to hydrogen peroxide, and similarly, catalase (CAT) converts hydrogen peroxide into molecular oxygen and water. Non-enzymatic antioxidants include vitamins, such as ascorbic acid, α-tocopherol, β-carotene, glutathione and flavonoids, and micronutrient elements like zinc and selenium [Polidori *et al.*, 2001]. Thus, active research is being undertaken to identify natural antioxidants with stronger pharmacological actions and fewer cytotoxic effects.

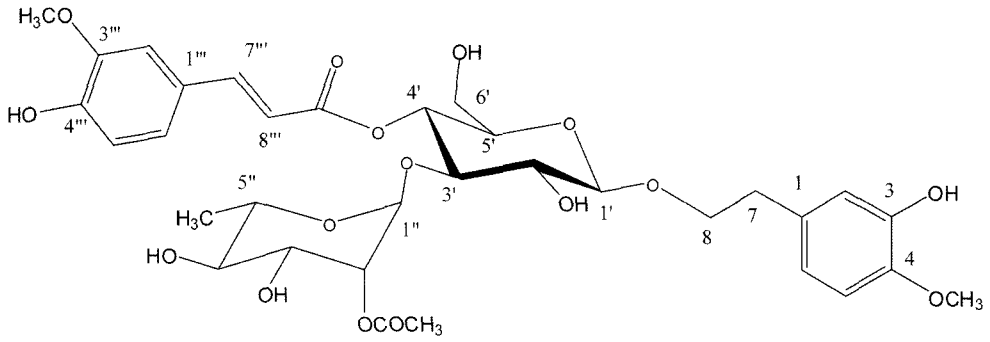
Clerodendron trichotomum Thunb., whose Japanese name is Kusagi, belongs to the Verbenaceae, which grows wild in fields and mountains in Korea, Japan and China [Ichi *et al.*, 1996]. The dried leaf and stem of *C. trichotomum,* which is called 'Chou Wu Tong (臭梧桐)', has diverse pharmacological activities, such as antihypertensive, sedative, analgesic, anti-rheumatoid and anti-inflammatory properties [Huang, 1993]. Several flavonoids [Morita *et al.*, 1977; Okigawa *et al.*, 1970], diterpenes [Kato *et al.*, 1971; Kawai *et al.*, 1998; Kawai *et al.*, 1999], blue pigments [Iwadare *et al.*, 1974], sterols [Kawano *et al.*, 1967] and phenylpropanoid glycosides [Kim *et al.*, 2001; Sakurai and Kato, 1983] have been isolated from *C. trichotomum.*

Many studies have focused mainly on the biological activities of phenolics, which are potent antioxidants and free radical scavengers [Marja *et al.*, 1999; Rice-Evans *et al.*, 1995]. However, little research has been done on

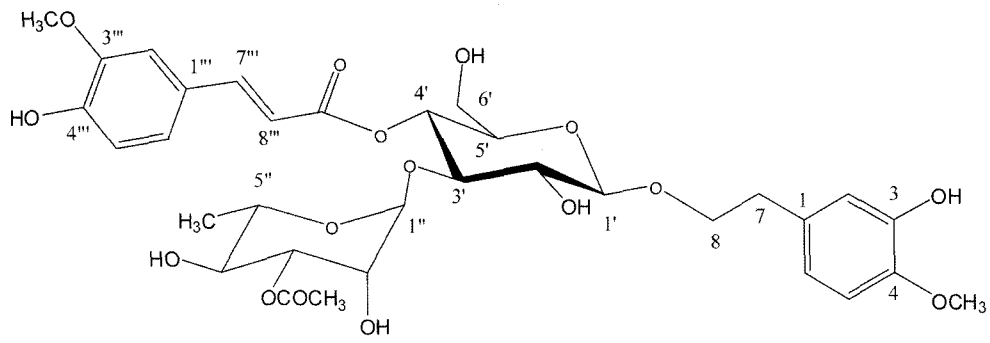
*Sam Sik Kang and Jin Won Hyun contributed equally to this study

*Corresponding authors
Phone: +82-02-740-8925; Fax: +82-02-743-3323
E-mail: sskang@snu.ac.kr
Phone: +82-064-754-3838; Fax:+82-064-726-4152
E-mail: jinwonh@cheju.ac.kr

Abbreviations: DCF-DA, 2',7'-dichlorofluorescin diacetate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; MPLC, medium pressure liquid chromatography; NAC, *N*-acetylcysteine; ROS, reactive oxygen species.



2″-acetylmartynoside



3″-acetylmartynoside

Fig. 1. Chemical structures of 2''-acetylmartynoside and 3''-acetylmartynoside.

phenolic compounds that occur in lipophilic extracts than those in hydrophilic extracts. Previously, we isolated lipophilic antioxidant, trichotomoside [Chae *et al.*, 2006], and our ongoing research for finding out antioxidant in *C. trichotomum* led us to isolate acetylmarthynosides. In this paper, we describe isolation and antioxidant activities of two lipophilic acetylmartynosides.

Materials and Methods

Plant material. The stems of *C. trichotomum* were collected on Sorok island, South Korea, during September 2002, and authenticated by Prof. KiHwan Bae of the College of Pharmacy, Chungnam National University, Chungnam, South Korea.

Instruments. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 instrument (300 MHz) using TMS as the internal standard. MPLC was performed using a YFLC-GR II equipment (Yamazen equipped with Pump 540, Prep · UV-10 UV detector and GR-202 gradient solvent supporter, Japan). For this, YFLC 30 cm-length of glass columns with inner diameter of 1.1 or 2.6 cm were used, packed with YFLC Gel, dp: 40 mm, pore size: 60Å. TLC was performed on silica gel $60F_{254}$ (Merck) and TLC spots were detected under UV lamp or by heating after spraying with 20% aqueous H_2SO_4.

Extraction and isolation. Dried stems of *C. trichotomum* (5 kg) were cut into small pieces and refluxed three times with MeOH. The MeOH extract was suspended in water and partitioned sequentially with n-hexane, CH_2Cl_2, EtOAc and n-BuOH. The CH_2Cl_2 extraction was divided into five fractions (Fr. A~Fr. E) by open column chromatography using the following solvent system; CH_2Cl_2 : MeOH (100 : 0 → 40 : 60). Fr. C (1.88 g) was chromatographed repeatedly on open column chromatography to give three fractions (Fr. C-1~Fr. C-3) using the following solvent system; n-hexane:EtOAc (20 : 80 → 10 : 90). Fr. C-2 was further separated by MPLC [CH_2Cl_2 : EtOAc(H_2O) : 2-propanol = 6 : 4 : 1] and solvent was delivered at 5 mL/min flow rate and 50 mL fractions were collected with a fraction collector (FR-50N), and finally purified by column chromatography over LiChroprep® RP-18 (40~63 μm, Art. 113900, Merck), with MeOH : H_2O (30 : 70), to give 2''-acetylmartynoside and 3''-acetylmartynoside. The chemical structures of 2''-acetylmartynoside and 3''-acetylmartynoside are shown in Fig. 1.

2''-Acetylmartynoside- Yellow amorphous powder. ^{1}H NMR (Me_2CO-d_6, 300 MHz) δ: 7.64 (1H, d, J = 15.9 Hz, H-7'''), 7.30 (1H, d, J = 2.1 Hz, H-2'''), 7.14 (1H, dd, J = 2.1, 8.4 Hz, H-6'''), 6.86 (1H, d, J = 8.4 Hz, H-5'''), 6.83 (1H, d, J = 8.4 Hz, H-5), 6.75 (1H, d, J = 2.1 Hz, H-2), 6.67 (1H, dd, J = 2.1, 8.4 Hz, H-6), 6.40 (1H, d,

Table 1. ^{13}C NMR data for 2''-acetylmartynoside and 3''-acetylmartynoside (δ, Me_2CO-d_6)

	No.	δ_C	
		2''-acetylmartynoside	3''-acetylmartynoside
	1	132.6	132.5
	2	116.7	116.7
	3	146.8	146.8
	4	146.7	146.7
	5	112.5	112.5
	6	120.7	120.7
	7	36.2	36.2
	8	71.4	71.4
glucose	1'	103.8	103.7
	2'	75.8	75.6
	3'	79.2	80.2
	4'	70.2	70.1
	5'	75.9	75.9
	6'	62.4	62.2
rhamnose	1''	102.2	99.3
	2''	69.6	73.2
	3''	73.2	70.2
	4''	70.6	73.7
	5''	69.7	69.6
	6''	18.6	18.4
	1'''	127.3	127.4
	2'''	111.3	111.3
	3'''	148.7	148.7
	4'''	150.2	150.2
	5'''	116.0	116.0
	6'''	124.2	124.2
	7'''	147.2	147.2
	8'''	115.2	115.2
	9'''	167.1	167.0
	2''-$COCH_3$	21.0	
	3''-$COCH_3$		20.9
	2''-$COCH_3$	170.3	
	3''-$COCH_3$		170.8
	4-OCH_3	56.3	56.3
	3'''-OCH_3	56.3	56.3

J = 15.9 Hz, H-8'''), 5.25 (1H, d, J = 1.5 Hz, H-1''), 4.40 (1H, d, J = 7.0 Hz, H-1'), 3.90 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.2-4.3 (12H, m, H-2'~6', H-2''~5'', H-8), 2.78 (2H, br. t, J = 7.8 Hz, H-7), 1.96 (3H, s, 2''-$COCH_3$), 1.13 (3H, d, J = 6 Hz, H-6''). ^{13}C-NMR (Me_2CO-d_6, 75 MHz): see Table 1.

3''-Acetylmartynoside- Yellow amorphous powder. ^1H NMR (Me_2CO-d_6, 300 MHz) δ: 7.63 (1H, d, J = 15.9 Hz, H-7'''), 7.3 (1H, d, J = 2.1 Hz, H-2'''), 7.14 (1H, dd, J = 2.1, 8.4 Hz, H-6'''), 6.86 (1H, d, J = 8.4 Hz, H-5'''), 6.83 (1H, d, J = 8.4 Hz, H-5), 6.76 (1H, d, J = 2.1 Hz, H-2), 6.68 (1H, dd, J = 2.1, 8.4 Hz, H-6), 6.40 (1H, d, J = 15.9 Hz, H-8'''), 5.25 (1H, d, J = 1.5 Hz, H-1''), 4.42 (1H, d, J = 7.8 Hz, H-1'), 3.90 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.2-4.3 (12H, m, H-2'~6', H-2''~5'', H-8), 2.79 (2H, br. t, J = 7.5 Hz, H-7), 1.99 (3H, s, 3''-$COCH_3$), 1.14 (3H, d, J = 6.3 Hz, H-6''). ^{13}C-NMR (Me_2CO-d_6, 75 MHz): see Table 1.

Cell culture. V79-4 Chinese hamster lung fibroblast cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained at 37°C in an 5% CO_2 humidified incubator in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/mL) and penicillin (100 unit/mL).

Chemicals. The DPPH radical and DCF-DA were purchased from Sigma, St. Louis, USA. Other chemicals and reagents were of analytical grade.

Intracellular ROS measurement. The DCF-DA method was used to detect intracellular ROS. DCF-DA diffuses into cells, where is hydrolyzed by intracellular esterase to polar 2',7'-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog is trapped in cells and can be oxidized to the highly fluorescent 2',7'-dichlorofluorescein by intracellular oxidants [Rosenkranz *et al.*, 1992]. V79-4 cells were seeded in a 96 well plate at a concentration of 1.0×10^5 cells/mL, and 16 h after plating were treated with acetylmartynosides (10 μg/mL), and 30 min later 1 mM H_2O_2 was added to the plate. Cells were then incubated for an additional 30 min at 37°C. The fluorescence of 2',7'-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission, using a Perkin Elmer LS-5B spectrofluorometer.

DPPH radical scavenging activities. Acetylmartynosides (10 μg/mL) were added to a 1.0×10^{-4} M solution of DPPH in MeOH, and the reaction mixture shaken vigorously. Five hours later amounts of DPPH remaining were determined by measuring absorbance at 520 nm [Lo *et al.*, 2004].

Statistics. All the measurements were made in triplicate. The results were subjected to an analysis of the variance (ANOVA) using the Turkey test to analyze the difference. $p < 0.05$ were considered significantly.

Results and Discussion

The CH_2Cl_2 extract of *C. trichotomum* was subjected to sequential column chromatography on silica gel and MPLC to yield 2''-acetylmartynoside and 3''-acetylmartynoside. They were obtained as a yellow amorphous powder. All proton and carbon chemical shifts were assigned and confirmed by the direct comparison of

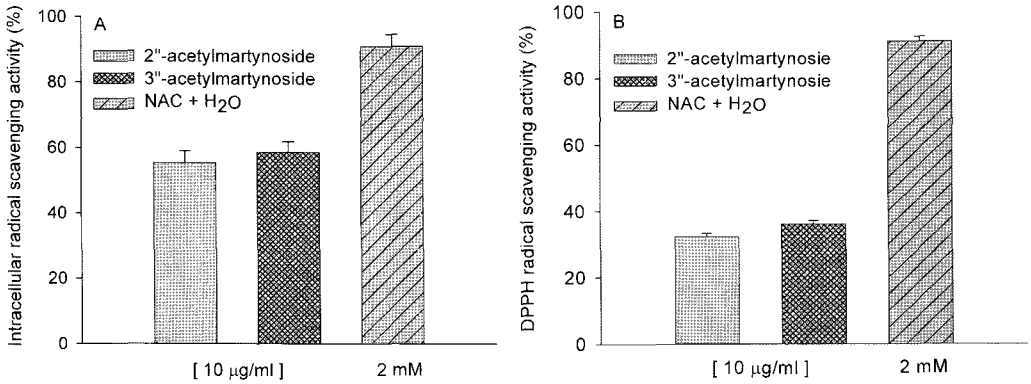


Fig. 2. Scavenging effects of 2"- and 3"-acetylmartynoside from *C. trichotomum* on intracellular ROS (A) and DPPH radical (B). Intracellular ROS was detected using the DCF-DA method. The amount of DPPH radicals were detemined spectrophotometrically. All experiments were performed in triplicate, and values are expressed as means ± SDE. NAC was used as positive control.

spectral data reported in the literature [Leitao *et al.*, 1994], and they were isolated for the first time from this plant.

In mammals ROS are constantly produced by the normal oxidative and reductive processes of mitochondria, but are also produced by immune system reactions to foreign antigens, and by external stimuli, such as exposure to UV or noxious chemicals. To prevent the potentially deleterious effects of ROS an anti-oxidant defense system comprised of antioxidant enzymes, such as, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, and various anti-oxidant chemicals, such as vitamins C and E, uric acid and bilirubin, constantly removes ROS. Thus, a balance between the generation of ROS and its removal is created, which is essential for maintaining cellular functions. However, when ROS production is excessive or the antioxidant system is suppressed, cells are attacked by ROS (a process often referred to as "oxidative stress"). Moreover, many diseases cause oxidative stress, and many studies have addressed the treatment of such diseases via the administration of anti-oxidants. For example, diabetic patients are subjected to levels of oxidative stress that are much higher than those found in normal subjects, and some of the complications of diabetes are relieved by anti-oxidant treatment [Ludvigsson, 1993]. Moreover, such findings have generated much research interest in natural antioxidants. For this reason, the antioxidant properties of 2"-acetylmartynoside and 3"-acetylmartynoside have been characterized.

Radical scavenging activity of 2"-acetylmartynoside and 3"-acetylmartynoside. The antioxidant activities of 2"-acetylmartynoside and 3"-acetylmartynoside, from *C. trichotomum,* were evaluated by the intracellular ROS and DPPH free radical scavenging activities. Antioxidant activities of 2"- and 3"-acetylmartynosides were evaluated by intracellular ROS and DPPH radical scavenging activities.

Glucose was used as negative control and the intracellular ROS and DPPH scavenging activity were 4.6% and 5.8%, respectively (data were not shown). ROS scavenging activity was expressed as percent inhibition and calculated using the following formula: % ROS scavenging activity = (control OD – sample OD/control OD) × 100.

DPPH radical scavenging activity was also expressed as percent inhibition as compared to control and calculated by formula as followed. % DPPH scavenging activity = (control OD – sample OD/control OD) × 100.

The intracellular ROS scavenging activities of 2"-acetylmartynoside and 3"-acetylmartynoside were 55.3% and 58.5% in 10 μg/mL (Fig. 2A), respectively. Moreover, both compounds at 10 μg/mL effectively inhibited the intracellular ROS almost 60% compared to the NAC used as a positive control (91%). The intraclellular ROS scavenging activities of 2"-acetylmartynoside and 3"-acetylmartynoside were substancially higher than their DPPH radical scavenging activities (Fig. 2B). 2"-Acetylmartynoside and 3"-acetylmartynoside at 10 μg/mL scavenged DPPH radical by 32.3% and 36.1%, respectively, which might suggest that they do not act as simple radical quencher, but rather as inducers of antioxidant defense system, by increasing the expressions of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase. Due to the lipophilic character of these acetylmartynosides, they are probably poor radical quenchers because of their relatively low solubilities in hydrophilic DPPH assay environment. An evaluation of the contribution made by acetylmartynosides to lipophilic antioxidant activity is probably warranted, though this will require the development of an alternative assay

system. In particular, the effects of these acetylmartynosides on antioxidant enzyme expressions and activations should be examined. Taken together, our results suggest that 2"-acetylmartynoside and 3"-acetylmartynoside showed antioxidant activity in terms of intracellular ROS scavenging effects.

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References

Bravo L (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev* **56**, 317-333.

Chae S, Kang KA, Kim JS, Hyun JW, and Kang SS (2006) Trichotomoside: a new antioxidative phenylpropanoid glycoside from *Clerodendron trichotomum*. *Chem Biodivers* **3**, 41-48.

Halliwell B and Gutteridge JMC (1998) *Free Radicals in Biology and Medicine*, pp. 22-35, Oxford University Press, New York.

Huang KC (1993) *The Pharmacology of Chinese Herbs*, pp. 71-72, CRC press, USA.

Ichi T, Shimizu T, and Yoshihira K (1996) *Biotechnology in Agriculture and Forestry, vol. 37. Medicinal and Aromatic Plants IX*, p. 108, Springer, Berlin Heidelberg.

Iwadare S, Shizuri Y, Sasaki K, and Hirata Y (1974) Isolation and structure of trichotomine and trichotomine G1. *Tetrahedron* **30**, 4105-4111.

Kawai K, Amano T, Nishida R, Kuwahara Y, and Fukami H (1998) Clerodendrins from *Clerodendron trichotomum* and their feeding stimulant activity for the turnip sawfly. *Phytochem* **49**, 1975-1980.

Kawai K, Nishida R, and Fukami H (1999) Clerodendrin I, a new neoclerodane diterpenoid from *Clerodendron trichotomum*. *Biosci Biotechnol Biochem* **63**, 1795-1797.

Kawano N, Miura H, and Kamo Y (1967) Studies on the components of *Clerodendron trichotomum* Thumb. II. *Yakugaku Zasshi* **87**, 1146-1148.

Kim HJ, Woo ER, Shin CG, Hwang DJ, Park H, and Lee YS (2001) HIV-1 integrase inhibitory phenylpropanoid glycosides from *Clerodendron trichotomum*. *Arch Pharm Res* **24**, 286-291.

Leitao SG, Kaplan MA, and delle Monache F (1994) Phenylpropanoid glucosides from *Aegiphila obducta*. *J Nat Prod* **57**, 1703-1707.

Lo SF, Nalawade SM, Mulabagal V, Matthew S, Chen CL, Kuo CL, and Tsay HS (2004) *In Vitro* Propagation by Asymbiotic Seed Germination and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Studies of Tissue Culture Raised Plants of Three Medicinally Important Species of *Dendrobium*. *Biol Pharm Bull* **27**, 731-735.

Ludvigsson J (1993) Intervention at diagnosis of type I diabetes using either antioxidants or photopheresis. *Diabetes Metab Rev* **9**, 329-336.

Marja PK, Anu IH, Heikki JV, Jussi-Pekka R, Kalevi P, Tytti SK, and Marina H (1999) Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem* **47**, 3954-3962.

Morita N, Arisawa M, Ozawa H, Chen CS, and Kan WS (1977) Clerodendroside, a new glycoside from the leaves of *Clerodendron trichotomum* Thunb. var. *fargesii* Rehd. *Yakugaku Zasshi* **97**, 976-979.

Okigawa M, Hatanaka H, and Kawano N (1970) A new glycoside, acacetin-7-glucurono-(1 → 2)-glucuronide from the leaves of *Clerodendron trichotomum*. *Tetrahedron Lett* **33**, 2935-2936.

Polidori MC, Stahl W, Eichler O, Niestroj I, and Sies H (2001) Profiles of antioxidants in human plasma. *Free Radic Biol Med* **30**, 456-462.

Rice-Evans C, Miller NJ, Bolwell GP, Bramley PM, and Pridham JB (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radic Res* **22**, 375-383.

Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W, and Zlabinger GJ (1992) A microplate assay for the detection of oxidative products using 2',7'-dichlorofluorescin-diacetate. *J Immunol Meth* **156**, 39-45.

Sakurai A and Kato T (1983) A new glycoside, kusaginin isolated from *Clerodendron trichotomum*. *Bull Chem Soc Jpn* **56**, 1573-1574.