Cognitive Improving and Cerebral Protective Effects of Acylated Oligosaccharides in *Polygala tenuifolia*

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We studied the cognitive improving and cerebral protective constituents in the roots of *Polygala tenuifolia* WILLDENOW, a well-known Chinese traditional medicine prescribed for amnesia, neurasthenia, palpitation, nocturnal emission and insomnia. Tenuifoliside B (1), which is one of the acylated oligosaccharides in the roots of *P. tenuifolia*, showed the cerebral protective effect on potassium cyanide (KCN)-induced anoxia in mice, widely used as an animal model for cerebrovascular disease, and also had an ameliorative effect on the scopolamine-induced impairment of performance in passive avoidance task in rats. Compound 1 significantly enhanced oxotremorine-induced tremors in mice, suggesting that it ameliorated the scopolamine-induced impairment of passive avoidance response by enhancing the cholinergic system. These findings show that compound 1 has cognitive improving and cerebral protective effects.

Key words *Polygala tenuifolia*; tenuifoliside B; memory; passive avoidance performance; potassium cyanide (KCN); anoxia

Senile dementia of the Alzheimer type and multi-infarct dementia are considered to be major problems of contemporary societies. In traditional Chinese medicine, Polygalae Radix (Japanese name: Onji), the root of *Polygala tenuifolia* WILLDENOW (Polygalaceae), is prescribed for amnesia, neurasthenia, palpitation, noctural emission and insomnia.[1] According to the Chinese Materia Medica, the root is guessed to have a special effect upon the will and mental powers, improving understanding and strengthening the memory.

There have been numerous studies regarding the reputed memory-enhancing potential of the roots of *P. tenuifolia*. DX-9368, which is composed of four herbs (*Panax ginseng*, *P. tenuifolia*, *Acorus gramineus* and *Poria cocos*), ameliorated the ethanol- and scopolamine-induced memory impairment in mice.[2] It is reported by Yabe *et al.* that the water extract of this plant up-regulates choline acetyltransferase (ChAT) activity and increases NGF secretion *in vitro*.[3] Recently, Egashira *et al.* reported that the water extract improved the scopolamine-induced impairment of passive avoidance response and enhanced oxotremorine-induced tremors in mice.[4] There are few reports, however, on the active constituents involved in the reputed memory-enhancing potential of the roots of *P. tenuifolia*, and we therefore studied these cognitive improving constituents. In this paper, we report that tenuifoliside B (**1**)[5] showed a cerebral protective effect on potassium cyanide (KCN)-induced anoxia and an ameliorative effect on the scopolamine-induced impairment of passive avoidance response in rats.

It is well known that a decrease in oxygen supply to the brain (hypoxia) depresses cerebral function in experimental animals and humans[6,7] and that memory and learning are impaired by hypoxia in animals and humans.[8,9] The KCN anoxia model is widely used in preclinical evaluations of drugs for the treatment of cerebrovascular disorders.[6,10,11] Therefore, we used this model in addition to the scopolamine-induced impairment of passive avoidance performance as screening method.

MATERIALS AND METHODS

Extraction and Fractionation of *P. tenuifolia* As shown in Fig 1, the dried roots (9.4 kg) of *P. tenuifolia* from Shanxi Province in China (purchased from Yamamoto Yakuhin Kogyo Co., Ltd., Tokyo) were pulverized and extracted twice with boiling MeOH (50 l) for 2 h. The concentrated MeOH extract (PM) (2.21 kg) was dissolved in H_2O and successively extracted with ether and *n*-BuOH. These layers were concentrated to give an ethereal soluble fraction (PE, xanthone-containing fraction) (398 g), an *n*-BuOH soluble fraction (902 g), and a water soluble fraction (PW) (609 g), respectively. The *n*-BuOH soluble fraction was chromatographed on silica gel [Kieselgel 60, 230—400 mesh (Merck)] (5 kg) with EtOAc–MeOH (4 : 1) and then with MeOH. Concentration of elutions with EtOAc–MeOH (4 : 1) and MeOH afforded the acylated oligosaccharide-containing fraction (PA) (468 g) and saponin-containing fraction (PS)

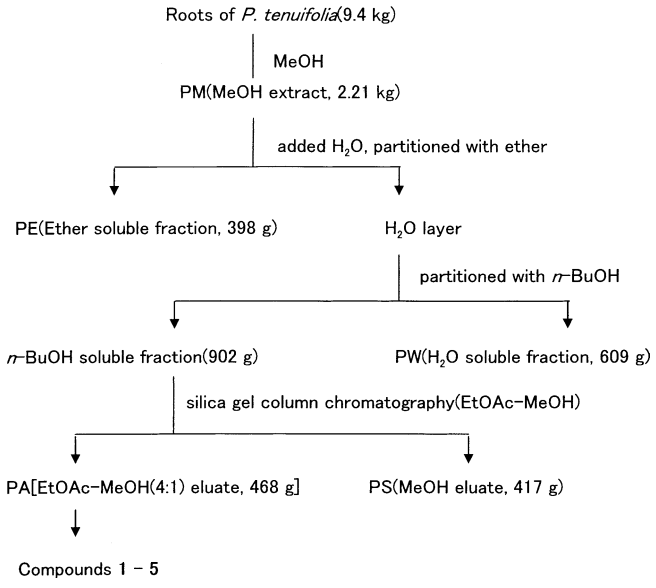

Fig. 1. Extraction and Fractionation of the Roots of *Polygala tenuifolia*

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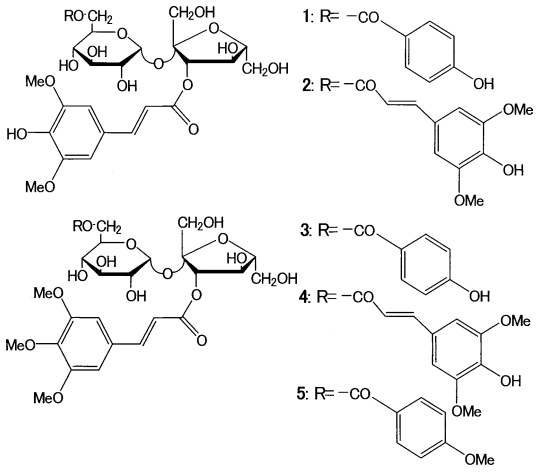

Fig. 2. Chemical Structures of Compounds **1**—**5**

(417 g), respectively. These fractions were checked by TLC [Kieselgel 60 F_{254} (Merck precoated plate) in $CHCl_3$–MeOH–H_2O (30 : 9 : 1) and *n*-BuOH–AcOH–H_2O (4 : 1 : 5) upper layer] and HPLC [column, TSK GEL ODS-80TS (4.6 i.d.×250 mm); mobile phase, CH_3CN–0.05% H_3PO_4 (15 : 85) (0—10 min) and CH_3CN–0.05% H_3PO_4 (15 : 85→65 : 35) (10—45 min, linear gradient); column temp., 40 °C; flow rate, 1.0 ml/min; detection, UV 300 nm; instrument, Shimadzu LC-10A].

Separation of Compounds 1—5 Purification of active fraction (PA) by repeated silica gel column chromatography and preparative thin layer chromatography [Kieselgel 60 F_{254} (Merck precoated plate)] with a mixture of $CHCl_3$–MeOH gave compounds **1** (13.38 g), **2** (10.09 g), **3** (11.59 g), **4** (1.78 g), and **5** (0.26 g) as major components of this fraction. Compounds **1**—**4** were identified as tenuifoliside B, 3,6′-disinapoylsucrose, tenuifoliside A, and tenuifoliside C, respectively, by direct comparison ($[\alpha]_D$, IR, MS, ^{1}H- and ^{13}C-NMR spectra) with the authentic samples.[5] Compound **5** was identified as β-D-[3-*O*-(3,4,5-trimethoxycinnamoyl)]-fructofuranosyl-α-D-[6-*O*-(4-methoxybenzoyl)]-glucopyranoside by comparing its $[\alpha]_D$, IR, MS, ^{1}H- and ^{13}C-NMR spectral data with the literature[12] and by analysis of the heteronuclear multiple bond connectivity (HMBC) spectrum.

Drugs Scopolamine hydrobromide, oxotremorine hydrochloride, physostigmine hemisulfate and tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride: THA) were purchased from Sigma-Aldrich Fine Chemicals Japan Co., Ltd., Tokyo and dissolved in 0.9% physiological saline.

Animals All animal experiments were performed in accordance with our institutional guidelines after obtaining the permission of the Laboratory Animal Committee. The animals used were male ICR strain mice, male ddY strain mice and male Wistar rats purchased from Charles River Japan (Atsugi, Japan). The animals were housed in groups of 5 per plastic cage and allowed access to water and standard laboratory food *ad libitum*. They were housed in a facility at a temperature of 23±2 °C, humidity of 55±5% and controlled lighting with lights on from 7:00 to 19:00.

Effect on KCN-Induced Anoxia in Mice Male ICR strain mice (5 weeks old) were used. Histotoxic anoxia was produced by an intravenous injection (i.v.) of 2 mg/kg KCN (0.1 ml/10 g). The time (coma time: s) until a disappeared righting reflex after KCN injection appeared was recorded. Test drugs were orally administered 60 min before KCN injection. All test drugs were suspended in 1% Tween 80 and given to mice at a dose of 0.1 ml/10 g. Control mice were given the vehicle orally.

Effect on Scopolamine-Induced Impairment of Passive Avoidance Performance Experiment 1: The MeOH extract (PM), PE fraction and PA fraction were tested for their effect on scopolamine-induced decrease of retention of passive avoidance in mice. Male ddY strain mice (5 weeks old) were used. The experiment was carried out using a step-through passive avoidance response apparatus (Neuroscience Inc., Tokyo). In a dark chamber, electric stimulation was given from the grid on the floor. The two chambers were divided with a movable guillotine-type door. The experiments consisted of training and test sessions. In the training session, a mouse was placed in the illuminated compartment and allowed 1 min or 3 min for habituation. The guillotine door was opened, then was closed immediately after the animal entered the dark compartment, and 10 s later an electric shock (200 V, 0.01 mA, 2.0 s) was delivered through the grid floor. Shock sensitivity (jump and squeal responses) was recorded when the shock was delivered. In the test session held 24 h after the training session, we used only mice that had jumped and squealed well. Each mouse was again placed in the illuminated compartment. The step-through latency before entering the dark compartment was measured in both sessions. The cut-off time was 300 s. Scopolamine (0.2 mg/kg) was administered to mice intraperitoneally (i.p.) 15 min before training, to impair memory registration. Test drugs were suspended in 1% Tween 80 and given orally 60 min before the retention test; physostigmine (0.25 mg/kg, i.p.) was given 30 min before the test. The escape latency was measured during a 300 s observation.

Experiment 2: Male Wistar strain rats weighing 200—300 g (8 weeks old) were used. The same step-through passive avoidance response apparatus as experiment 1 was used. As soon as a rat entered the dark compartment, a footshock (1 mA, 3.0 s) was given. The latency to enter the dark compartment was recorded. Scopolamine (1 mg/kg i.p.) was administered 30 min before the acquisition trial. Immediately after the footshock administration, compound **1** and tacrine (THA) were given to rats orally. Twenty-four hours later, a retention test was administered and the escape latency was measured during a 300 s observation.

Effect of on Oxotremorine-Induced Tremor in Mice Male ddY strain mice (5 weeks old) were used. Immediately after administration of oxotremorine (0.3 mg/kg, i.p.), each mouse was placed in a plastic container (10×30×30 cm), and the intensity of the tremors was recorded according to the following scores: 0, no abnormal behavior was observed; 1, intermittent slight tremors; 2, occasional moderate tremors as well as intermittent slight tremors; 3, persistent moderate tremors; 4, persistent severe tremors. THA and compound **1** were administered orally 50 min before the injection of oxotremorine (10 mice per group). Observation of the tremors was made at 10 min intervals starting 30 min after the oxotremorine treatment, and the intensity of the tremors was determined as the mean of the total score for 10—20 min.

Statistical Analysis The results are expressed as the mean±S.E.M. The statistical analysis of the data for KCN-

induced anoxia was performed by one-way ANOVA followed by Fischer's *post hoc* test. The Mann–Whitney *U*-test for the passive avoidance response and Wilcoxon's rank-sum test for oxotremorine-induced tremors were used to assess the differences in values between groups.

RESULTS

Effect on KCN-Induced Anoxia in Mice When the PE, PA , PS and PW fractions from the MeOH extract were tested for effects on KCN-induced anoxia in mice, only the PA fraction shortened the coma time significantly as shown in Table 1. TLC analyses [silica gel plates in $CHCl_3$–MeOH–H_2O (30 : 9 : 1)] revealed that the acylated oligosaccharides **1**—**5** were the major constituents of the PA fraction. Effects of the acylated oligosaccharides **1**—**5** on KCN-induced anoxia are shown in Table 2. Compounds **1** (3—10 mg/kg) and **2** (100 mg/kg) shortened the coma time significantly.

Effect on Scopolamine-Induced Impairment of Passive Avoidance Performance Experiment 1: The MeOH extract (PM), PE fraction, and PA fraction were tested in this examination. The PS (saponin-containing fraction) was not tested for because this fraction cased death at a dose of 100 mg/kg, *p.o.* The PW fraction was also not tested because it had no protective effect against the KCN-induced anoxia. As shown in Table 3, the load of scopolamine produced amnesia and the scopolamine control group shortened latency compared to the non-scopolamine control group. Scopolamine-induced decrease of the retention of passive avoidance was ameliorated by treatment with the PM (50 mg/kg, *p.o.*) and PA fractions (25 mg/kg, *p.o.*) as well as physostigmine (0.25 mg/kg, i.p.) whose ameliorative effect on scopolamine-induced impairment of passive avoidance responce is reported.[13] These results show that the ameliorating constituents against scopolamine-induced impairment are contained in the PA fraction. Thus, compound **1** which had showed significant cerebral protective effect was tested for its effect on scopolamine-induced amnesia in a passive avoidance task.

Experiment 2: As shown in Table 4, the load of scopolamine produced amnesia and the scopolamine control group shortened latency compared to the non-scopolamine control group. Compound **1** (3—10 mg/kg, *p.o.*) caused significant prolongation in the escape latencies in a dose-dependent manner as did THA (10 mg/kg, *p.o.*) whose ameliorative effect on scopolamine-induced amnesia in a passive avoidance task was reported.[14] These results suggest that compound **1** is one of the ameliorative constituents on scopolamine-induced passive avoidance failure in *P. tenuifolia*.

Table 1. Effect of Fractions of PM on the KCN-Induced Anoxia in Mice

Treatment	Dose (mg/kg, *p.o.*)	Coma time (s)
Control	—	296.3±42.9
PE	100	228.4±49.2
	500	194.8±54.2
Control	—	242.6±34.7
PA	100	175.0±40.1
	500	144.5±26.1*
Control	—	247.1±34.0
PS	10	251.2±31.1
	100	194.2±54.2
Control	—	126.8±25.4
PW	100	136.9±28.3
	500	144.9±26.5

Each value represents the mean±S.E. (n=15). * p<0.05 compared with the control value.

Table 2. Effect of Compounds **1**—**5** on the KCN-Induced Anoxia in Mice

Treatment	Dose (mg/kg, *p.o.*)	Coma time (s)
Control	—	280.4±33.8
Compound **1**	3	186.6±26.8*
	10	150.4±30.6*
	100	233.1±39.6
Control	—	315.9±45.2
Compound **2**	10	234.1±37.8
	100	197.0±31.6*
Control	—	136.7±23.2
Compound **3**	100	154.3±26.1
Control	—	217.2±33.4
Compound **4**	10	221.6±43.8
	100	174.7±28.5
Control	—	206.3±40.2
Compound **5**	10	202.3±41.4
	100	184.6±35.0

Each value represents the mean±S.E. (n=10). * p<0.05 compared with the control value.

Table 3. Effect of Fractions of PM on Scopolamine-Induced Impairment of Passive Avoidance Performance

Treatment	Dose (mg/kg)	Scopolamine	Latency (s)
Control (saline)		—	282.7±13.8
Control (1% Tween)		+	63.9±22.9
Physostigmine	0.25	+	126.5±51.7*
PM	50	+	96.3±32.4*
PE	25	+	65.2±31.3
PA	25	+	120.0±32.8*

Each value represents the mean±S.E. (n=10). * p<0.05 compared with the control (scopolamine-treated group) value (Mann–Whitney *U*-test).

Table 4. Effect of Compound **1** on Scopolamine-Induced Impairment of Passive Avoidance Performance

Treatment	Dose (mg/kg)	Scopolamine	Latency (s)
Saline		—	248.8±35.4
Control (saline)		+	63.4±36.0†
Compound **1**	1	+	79.5±43.7
Compound **1**	3	+	204.0±46.6*
Compound **1**	10	+	300.0±0.0**
THA	3	+	140.0±48.8
THA	10	+	188.4±43.8*

Each value represents the mean±S.E. (n=8). † p<0.05 compared with the non scopolamine-treated group, * p<0.05, ** p<0.01, compared with the control (scopolamine-treated group) value (Mann–Whitney test).

Table 5. Effect of Compound **1** on Oxotremorine-Induced Tremors in Mice

Treatment	Dose (mg/kg)	Score±S.E.
Control (saline)		1.3±0.2
THA	10	2.6±0.2***
Compound **1**	3	2.5±0.2**
Compound **1**	10	2.4±0.3**

The intensity of the tremors was determined as the mean of the total score for 10—20 min. Each value represents the mean±S.E. (n=10). ** p<0.01, *** p<0.001 compared with the control value.

Table 6. Ingredients of Kami-kihi-to, Ninjin-yoei-to and Kami-untan-to

Composition	Kami-kihi-to	Ninjin-yoei-to	Kami-untan-to
Angelicae Radix (root of *Angelica acutiloba* Kitagawa)	2.0 g	4.0 g	
Astragali Radix (root of *Astragalus membranaceus* Bge.)	3.0 g	1.5 g	
Atractylodes Lanceae Rhizome (root of *Atractylodes lancea* DC.)	3.0 g		
Atractylodes Rhizoma (root of *Atractylodes japonica* Koizumi)		4.0 g	
Aurantii Fructus Immaturus (immature fruit of *Citrus aurantium* L.)			3.0 g
Aurantii Nobilis Pericarpium (peel of *Citrus unshiu* Markov.)		2.0 g	3.0 g
Bupleuri Radix (root of *Bupleurum falcatum* L.)	3.0 g		
Cinnamomi Cortex (bark of *Cinnamomum cassia* Bl.)		2.5 g	
Gardeniae Fructus (fruit of *Gardenia jasminoides* Ellis)	2.0 g		
Ginseng Radix (root of *Panax ginseng* C. A. Mey.)	3.0 g	3.0 g	2.0 g
Glycyrrhizae Radix (root of *Glycyrrhiza uralensis* Fisch.)	1.0 g	1.0 g	2.0 g
Hoelen (sclerotium of *Poria cocos* Wolf)	3.0 g	4.0 g	3.0 g
Longanae Arillus (aril of *Euphoria longana* Lam.)	3.0 g		
Phyllostachysis caulis in Taeniam (stalk of *Phyllostachys nigra* Munro)			3.0 g
Pinella Tuber (tuber of *Pinellia ternate* Breit.)			5.0 g
Polygalae Radix (root of *Polygala tenuifolia* Willd.)	2.0 g	2.0 g	2.0 g
Paeoniae Radix (root of *Paeonia lactiflora* Pall.)		2.0 g	
Rehmanniae Radix (root of *Rehmannia glutinosa* Lib., var. *purpurea* Mak.)		4.0 g	2.0 g
Saussurea Radix (root of *Saussurea lappa* Clarke)	1.0 g		
Schisandra Fructus (fruit of *Schisandra chinensis* Baill.)		1.0 g	
Scrophulariae Radix (root of *Scrophularia ningpoensis* Hemsley)			2.0 g
Zingiberis Rhizoma (root of *Zingiber officinale* Rosc.)	1.0 g		0.5 g
Zizyphi Fructus (fruit of *Zizyphus jujuba* Mill. var. *spinosa* (Bunge) Huex H. F. Chou)	2.0 g		2.0 g
Zizyphi Spinosi Semen (seed of *Zizyphus jujuba* Mill.)	3.0 g		2.0 g

Effect of Compound 1 on Oxotremorine-Induced Tremor in Mice The effect of compound **1** on the oxotremorine-induced tremor in mice is shown in Table 5. Compound **1** (3—10 mg/kg, *p.o.*) significantly enhanced the tremor as did THA (10 mg/kg, *p.o.*) which is a muscarinic choline agonist.[15]

DISCUSSION

In the present study, we identified the cerebral protective constituents **1** and **2** against KCN-induced anoxic mice from Polygalae Radix (the roots of *Polygala tenuifolia*). We believe the reason compound **1** did not show the linear dose-dependency in this examination is that the examination was the first experiment on this compound and the dosage of 100 mg/kg was added to compare this compound with the other compounds. However, the optimal dosage of compound **1** is now thought to be 10 mg/kg, and it is guessed that there was an adverse effect to the dosage of 100 mg/kg. Therefore, a dosage of 10 mg/kg or less was used in the examination of compound **1** thereafter.

It was also found that compound **1** ameliorated the scopolamine-induced impairment of performance in passive avoidance task in rats. Further, the effect of compound **1** on tremors induced by oxotremorine which directly stimulates muscarinic acetylcholine M_1 receptors was investigated. Compound **1** at a dose of 3—10 mg/kg significantly enhanced the oxotremorine-induced tremors in mice as did THA.

It is generally accepted that the acute toxicity of KCN is largely due to the inhibition of mitochondrial cytochrome oxidase, producing cytotoxic anoxia.[16] Our data show that compound **1** suppresses the breakdown of cellular metabolism induced by the inhibition of cytochrome oxidase. Gibson *et al.* reported that KCN reduced potassium-stimulated synaptosomal acetylcholine release.[17] Nitta *et al.* reported that not only acetylcholine receptor but also dopamine receptor participated in the scopolamine-induced impairment of performance.[18] The data suggest that the ameliorating effect of compound **1** on scopolamine-induced impairment of performance relates to enhancement of the cholinergic system; but the mechanisms other than the acetylcholinergic system of this compound are unclear. The reason the efficacy of compound **1** (3—10 mg/kg, *p.o.*) was not shown in a dose-dependent manner in the examination on the oxotremorine-induced tremors is thought to be that the action on the cholinergic system of this compound reaches the saturated condition with a dosage of 3 mg/kg.

The PM and PA fractions showed the ameliorative effect on scopolamine-induced decrease of the retention of passive avoidance. Yabe *et al.* previously reported that the water extract of Polygalae Radix up-regulated the choline acetyltransferase (ChAT) activity and increased NGF secretion *in vitro*.[3] We considered that the PM and PA fractions might ameliorate the scopolamine-induced decrease of retention of passive avoidance by enhancing the central cholinergic system, *i.e.*, by enhancing of acetylcholine release and ChAT activity. The contents of compound **1** in the PA and PM fractions were 5.0% and 1.1%, respectively, by HPLC analyses. These contents seem to correspond to the dosage that the ameliorating effects of compound **1** (3 mg/kg), the PA (25 mg/kg) and PM (50 mg/kg) fractions on the scopolamine-induced impairment of performance were appeared, respectively.

Kami-kihi-to, Ninjin-yoei-to and Kami-untan-to are traditional Japanese herbal medicines (Kampo medicine) composed of fourteen, twelve and thirteen kinds of medicinal herbs as shown in Table 6, respectively, have been prescribed for senile dementia.[19—21] Recently, it has been reported that the clinical application of these Kampo medicines for patients with senile dementia of the Alzheimer type improved their memory related behavior.[22—26] All of them contain

Polygalae Radix, Ginseng Radix, Hoelen and Glycyrrhizae Radix as common herbs. There are some reports related to an anti-dementia effect of Polygalae Radix, Ginseng Radix, and Hoelen among the four medicinal herbs common to the above three Kampo medicines. Watanabe reported that the water extract of Ginseng Radix has improved the maintenance of memory by water maze examination.[27] Yabe *et al.* reported that the water extracts of Polygalae Radix and Hoelen increased ChAT activity of basal forebrain cells.[3] Tabata *et al.* reported that paeoniflorin in Paeoniae Radix ameliorated the adenosine A1 receptor-mediated impairment of passive avoidance performance excluding the medicinal herbs that are common to the above three Kampo medicines.[28] Longanae Arillus is used for the treatment of amnesia in herbal remedy prescriptions of Chinese and Japanese traditional medicine.[29]

Morever, Yabe *et al.* reported that the activities of incomplete formulas of Kami-untan-to excluding individual herbs were measured, and that only the ChAT activity of Kami-untan-to minus Polygalae Radix was a control level.[3] This finding suggests that Polygalae Radix plays an important role in the effect of Kami-untan-to on the central cholinergic system. The results demonstrated here, taken with others in the literature, indicate the possibility that Kami-kihi-to, Ninjin-yoei-to and Kami-untan-to may have potential therapeutic effect for the treatment of Alzheimer disease patients. Polygalae Radix and tenuifoliside B (**1**) seem to contribute to therapeutic effects of the above three Kampo medicines in cooperation with other herbs of these Kampo medicines.

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