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Abstract

The methanolic extract of sweet hydrangea leaf, processed leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO (*Saxifragaceae*), was found to show relaxant effects on contractions induced by high concentration of K^+ and noradrenaline in isolated rat aortic strips. Principal constituents, phyllodulcin and hydrangenol, and minor constituents, thunberginols A and B, showed vasorelaxant effects similar to those of two flavonoids (lueolin, quercetin) and a calmodulin inhibitor (W-7).

1. Introduction

Sweet hydrangea leaf, a natural medicine indigenous to Japan, is prepared from the leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO (Saxifragaceae) *via* several processing steps such as crumpling, fermentation and drying. This natural medicine is listed in the Japanese pharmacopoeia XVII and is used in confectionery, drinks and food as an oral refrigerant and a sweetening. Two dihydroisocoumarins, phyllodulcin (**1**) and hydrangenol (**2**), have been isolated as the major constituents of this natural medicine (the processed leaves), while their 8-*O*-glucosides were isolated from the non-processed leaves. These findings imply that, during the processing, the glucosides are hydrolyzed to the corresponding aglycones (**1** and **2**).[1]

This traditional herb was found at Edo period as a variation of *Hydrangea* genus, and has been used as a folk medicine in Japan, but its functional or pharmacological effects have not been clarified yet. As a part of our studies of the bioactive constituents of this herb, the methanolic (MeOH) extract of this natural medicine was found to show potent antiallergic, antibacterial, antioxidative, antiulcer, cholagoic effects.[2,3] We reported the isolation

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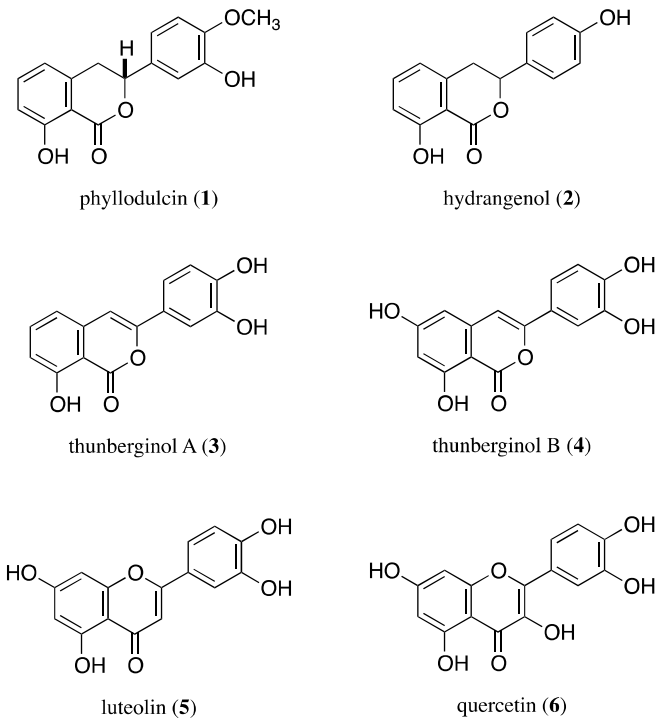

Fig. 1. *Chemical structures of* ***1****–****6***

and structural characterization of two isocoumarins [thunberginols A (**3**) and B (**4**)], three dihydroisocoumarins [thunberginols C, D, and E], a benzalphthalide [thunberginol F], three dihydroisocoumarin glucosides [thunberginol G 3'-*O*-glucoside, (+)- and (–)-hydrangenol 4'-*O*-glucoside], two phthalides [hydramacrophyllols A and B] as antiallergic and antibacterial principles of this herb. [4–12] Furthermore, two major constituents (**1** and **2** in Fig. 1) and hydrangeic acid were found to show antidiabetic effects *in vitro* and in KK-A^{y} mice, a type-2 diabetic mice.[13,14]

As a continuing study of this herb, to clarify whether this herb has a good effect on a cardiovascular system or not, we examined effects of the MeOH extract and its several constituents (**1**–**4**) (Fig. 1) on contractions of isolated rat aorta strips induced by high concentration (60 mM) of K^+ (High K^+) and noradrenaline (1 μM).

2. Material and Methods

2.1 Extraction and Isolation

The MeOH extract (20.3% from the leaves), phyllodulcin (**1**, isolation yield: 1.99% from the leaves), hydrangenol (**2**, 2.35%), and thunberginols A (**3**, 0.0086%) and B (**4**, 0.0016%) were prepared from sweet hydrangea leaves as described in our previous report.[4] Noradrenaline (L-(−)-norepinephrine (+)-bitartrate salt monohydrate) and quercetin (**6**) were purchased from Sigma-Aldrich, and luteolin (**5**) and other reagents were purchased from Wako Pure Chemicals Industries Ltd.

2.2 Vasorelaxtant effects

2.2.1 Tissue preparation

Male Sprague-Dawley rats (6–7 weeks old) were sacrificed by severing both carotid arteries under anesthesia, and the thoracic aorta was isolated and cut into helical strips (1.5–2.5 mm × 10–14 mm), and endothelium was removed and used in this study. Physiological salt solution containing NaCl (118.0 mM), KCl (4.7 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2.5 mM), $NaHCO_3$ (25.0 mM), and D-glucose (10.0 mM) was used in the investigation. The solution was aerated with a 95% O_2–5% CO_2 gas mixture and kept at 37 ˚C. To investigate the mechanical response, each preparation was suspended in an organ bath (6 mL) and subjected to an initial load of about 1 g. One hour equilibration period was allowed before initiation of the experiments. Contractions were measured isometrically *via* a force-displacement transducer (AD Instruments) and recorded on a polygraph. The experimental protocol was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

Table 1. *Vasorelaxant effects of the MeOH extract, compounds* ***1****–****6****, and W-7 on sustained contractions induced by High* K^+ *and noradrenaline in isolated rat thoracic aorta*

	Relaxation (%)					
	High K^+			Noradrenaline (1 μM)		
	10 μg/mL	30 μg/mL	100 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL
Control (vehicle)	2.4±1.1	3.6±1.1	14.7±1.7	0.7±1.1	3.8±1.3	3.3±1.7
MeOH ext.	10.7±3.5	20.9±4.1	63.2±4.7**	2.6±3.2	6.5±3.8	82.9±7.0**
	High K^+			Noradrenaline (1 μM)		
	10 μM	30 μM	100 μM	10 μM	30 μM	100 μM
Phyllodulcin (**1**)	4.9±2.1	16.0±5.4	69.8±12.0**	4.7±1.9	19.0±5.9	38.1±4.3**
Hydrangenol (**2**)	5.3±0.9	10.1±1.8	31.5±4.1**	0.3±0.2	0.5±0.5	18.7±3.7
Thunberginol A (**3**)	9.4±0.9	58.8±3.8**	88.1±1.4**	38.4±18.5*	100.0±0.0**	100.0±0.0**
Thunberginol B (**4**)	8.9±1.7	42.2±5.6**	100.0±0.0**	16.5±3.1	88.2±7.5**	100.0±0.0**
Luteolin (**5**)	9.8±2.9**	38.9±3.2**	96.7±3.0**	49.7±10.4**	85.5±8.7**	94.4±5.1**
Quercetin (**6**)	19.1±3.1**	50.8±6.2**	88.8±3.4**	47.7±12.7**	87.2±8.2**	100.0±0.0**
W-7	0.0±0.0	12.6±0.6	78.3±6.4**	5.8±2.9	32.5±9.1**	80.7±7.2**

Each value represents mean±S.E.M (n=4–8). Statistically significant from the control, * p<0.05, ** p<0.01.

2.2.2 Inhibitory effect on the contraction induced by noradrenaline or high K+

Experiments were performed according to our previous report with slight modification.[15] After equilibration, noradrenaline (final concentration: 1 μM) or 2 M KCl (0.18 mL, final concentration of K^+: *ca*. 60 mM) was added to the bath. Tissue samples were each washed 3 times and re-equilibrated after the contraction had reached the maximum level (0.6–1.2 g). Sustained contraction was induced again by the addition of noradrenaline or KCl, and then the test-compound was cumulatively applied at 10–100 μM (final conc.). The contractile response prior to the application of the tes-compound was taken as 100%. A calmodulin inhibitor (W-7), and two flavonoids (luteolin (**5**) and quercetin (**6**)) were used as reference compounds.

2.2.3 Statistical analysis

Values are expressed as the means±S.E.M. For statistical analysis, one-way analysis of variance followed by Dunnett's test for multiple comparison analysis was used. Probability (p) values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1 Vasorelaxant effect of MeOH extract from sweet hydrangea leaf on the contraction induced by noradorenaline and high K+ in isolated rat thoracic aorta

High K^+ (60 mM) and noradrenaline (1 μM) caused sustained contractions in isolated rat aortic strips (Fig. 2). After reaching maximal contractions, the test-compound, which was dissolved in dimethylsulfoxide (DMSO), was cumulatively added to the organ bath.

The MeOH extract showed significant vasorelaxant effects on the contractions at 100 μg/mL (Table 1). The major constituents, phyllodulcin (**1**) and hydrangenol (**2**) also moderately inhibited them. Although weaker than those of flavonoids (**5** and **6**), their effects were almost the same as those of calmodulin inhibitor W-7.

A flavonoid-rich diet has been associated with a lower incidence of cardiovascular diseases, probably because of the antioxidant and vasoactive properties of flavonoids. In fact, many flavonoids show vasorelaxing properties, although their mechanisms of action are different and often not completely clarified as yet.[16–18] In the present study, thunberginols A (**3**) and B (**4**), which were isolated as an antiallergic constituents from this herb, showed stronger inhibition and their potencies and efficacies approximated well to the both flavonoids (**5** and **6**) as shown in Table 1 and Fig. 2.

The important mechanism of vascular smooth muscle (VSM) contraction is shown in Fig. 3. Briefly, an increase in free intracellular Ca^{2+} results from either increased flux of Ca^{2+} into the cell through Ca^{2+}-channels or by release of Ca^{2+} from internal stores (*e.g.*, sarcoplasmic reticulum; SR). The free Ca^{2+} binds to calmodulin (CaM), and then Ca^{2+}-CaM activates myosin light chain kinase (MLCK) which phosphory-

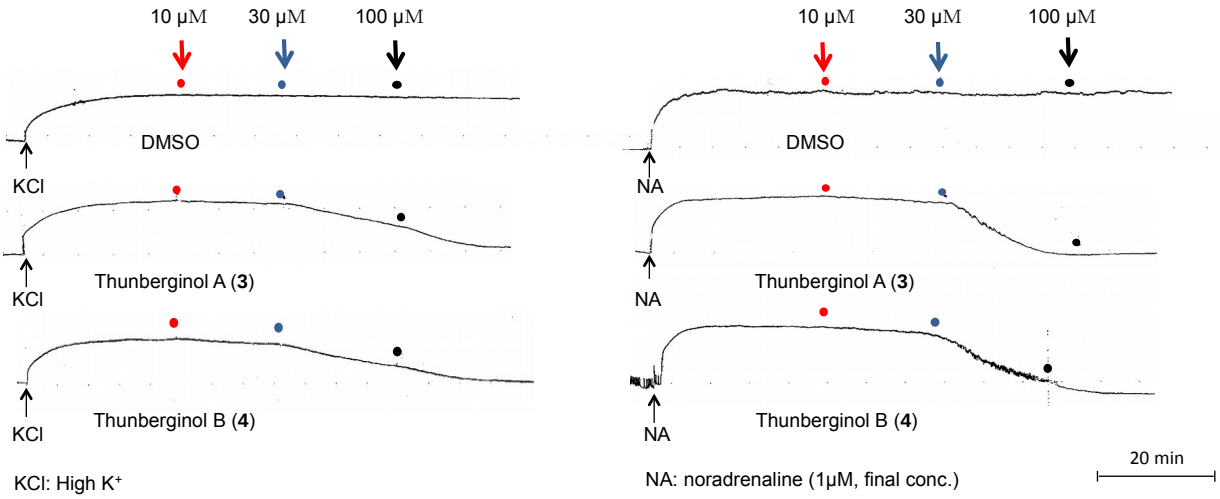

Fig. 2. *Vasorelaxant effects of thunberginols A (3) and B (4) on sustained contractions induced by High K^+ and noradrenaline in isolated rat thoracic aorta*

lates myosin light chains (MLC) in the presence of ATP. MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and then smooth muscles contract. Reduction in release of Ca^{2+} from the SR or reduction in Ca^{2+} influx into the cell, inhibition of MLCK by increased intracellular concentration of cAMP, and phosphatase-activated MLC dephosphorylation can reduce phosphorylation of MLC.[19]

The degree of MLC phosphorylation is regulated by G-protein-couple signal transduction pathways and by nitric oxide activation of guanylate cyclase and cGMP formation. As shown in Fig. 3, Gq-protein activation by noradrenaline (α_1-adrenoceptors), angiotensin II (AT_1 receptors), endothelin-1 (ET_A receptors), and vasopressin (V_1 receptors) stimulates SR release of calcium (IP_3 mediated) and activates Rho-kinase, which inhibits MLC phosphatase (MLCP). Both of these mechanisms enhance VSM contraction. Gs-protein activation by compounds such as adrenaline (β_2-adrenoceptors), adenosine (A_2 purinergic receptors) and prostacyclin (IP receptors) increase cAMP, which inhibits MLCK thereby reducing MLC phosphorylation and relaxing the VSM. Gi-protein activation by noradrenaline binding to α_2-adrenoceptors elicits contraction by reducing cAMP, which increases the activity of MLCK.[19]

High K^+-induced contractions in smooth muscle is known to be the result of an increase in Ca^{2+} influx through a voltage-dependent Ca^{2+} channel (L-type channel), and Ca^{2+}-blockers such as nifedipine selectively inhibit this contraction. However, noradrenaline activates release of Ca^{2+} from SR and a receptor-operated Ca^{2+} channel, but not the voltage-dependent Ca^{2+} channel. To date, we have examined effects of medicinal herbs and their constituents on High K^+ and noradrenaline-induced contractions in rat aortic strips as a screening test for vasodilating compounds.[15, 20–22] In the present study, the relaxant effects of the MeOH extract and the active constituents were observed in both (High K^+- and noradrenaline-induced) contractions, suggesting that their mechanisms of action may have been induced after the increase in intracellular Ca^{2+}levels.

Calmodulin (CaM) is an acidic protein considered to be the universal calcium sensor. Intracellular Ca^{2+} levels are mediated by CaM, thus controlling a myriad of physiological responses such as cell proliferation, endocytosis, cellular adhesion, protein turnover, and smooth muscle contractions. In the present study, the metnanolic extract, its active constituents (**1**–**4**), and flavonoids (**5**, **6**) significantly inhibited both contractions in a concentration-dependent manner similar to CaM inhibitor, W-7. In our previous study of anti-allergic effects of thunberginols A (**3**) and B (**4**), we reported that **4** and luteolin (**5**) showed a similar profile of mRNA expression and mode of action in RBL-2H3 cells.[12] The results in the present study and our previous study suggested that **4**, at least in part, has a mechanism similar to **5** in VSM, although further study is needed to clarify the mechanism.

In conclusion, the MeOH extract of sweet hydrangea leaf showed relaxant effects on contractions induced by High K^+ and noradrenaline in isolated rat aortic strips. Principal constituents (see Fig. 1), phyllodulcin (1) and hydrangenol (2), and minor constituents, thunberginols A (3) and B (4), showed vasorelaxant effects similar to those of luteolin (5), quercetin (6), and a calmodulin inhibitor, W-7.

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