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# IMMUNOMODULATORY ACTIVITY OF THUNBERGINOL A AND RELATED COMPOUNDS ISOLATED FROM HYDRANGEAE DULCIS FOLIUM ON SPLENOCYTE PROLIFERATION ACTIVATED BY MITOGENS

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**Abstract**: We investigated the immunomodulatory effects of antiallergic constituents from Hydrangeae Dulcis Folium, the processed leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO, on splenocyte proliferation in mice. Thunberginol A and hydrangenol significantly suppressed T lymphocyte proliferation induced by concanavalin A. Thunberginol A also suppressed B lymphocyte proliferation induced by lipopolysaccharide, but other constituents induced significant increases. These inhibitory effects of thunberginol A on splenocyte proliferation seemed to contribute to the suppressive effect on type IV allergy.


We have been searching for antiallergic principles in Hydrangeae Dulcis Folium (Amacha in Japanese), the processed leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO, using the Schults-Dale reaction on the guinea pig trachea chain and measurement of histamine release from rat peritoneal exudate cells caused by some histamine releasers. As a result, we found nine new antiallergic principles; thunberginols A (**2**), B (**3**) (isocoumarins) , C (**6**), D (**7**), E (**9**), G(**5**) (dihydroisocoumarins), and F (**10**) (benzylidenephthalide), and hydramacrophyllols A and B (phthalides) from this natural medicine.[1] The antiallergic activities of these compounds were more potent than those of hydrangenol (**4**)[2] and phyllodulcin (**8**)[2] which are principal constituents of Hydrangeae Dulcis Folium.[3] Among these compounds, the isocoumarins **2**, **3** and the benzilidenephtalide **10** showed more potent antiallergic activities than the other dihydroisocoumarins and phthalides. Especially, oral administration of **2** inhibited not only various type I allergic reactions *in vitro* but also the passive cutaneous anaphylaxis (PCA) reaction and asthmatic bronchoconstriction in rats induced by antigen-antibody reaction *in vivo*.[4] In addition, **2** suppressed type IV allergy reaction in mice which is known as delayed type hypersensitivity (DTH)[4]; i.e., **2** significantly inhibited the contact ear dermatitis induced by dinitrofluorobenzene by consecutive oral administration in the primary sensitizing phase. Oral administration of **2** also reduced the hind paw edema sensitized with sheep red blood cells (SRBC) in both sensitizing and inflammatory phases.[4] The antigen presenting cells (APC; macrophages, Langerhans's cells) and delayed type hypersensitivity T lymphocytes ($T_{DTH}$; mainly Th1, $CD4^+$), which are sensitized with specific antigens, are thought to play important roles in DTH reaction.[5] As **2** especially inhibited DTH reaction by administration in the sensitizing phase, it was suggested that this compound might have immunomodulatory actions on antigen processing by macrophages and T lymphocyte sensitization or activation. Various phytolectins are known as mitogens which show non-specific proliferative activity for lymphocytes. These mitogens are widely used to investigate the effects of immunosuppressants on lymphocyte activation. To clarify the effects of **2** on antigen-

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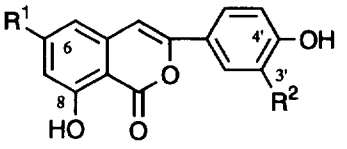
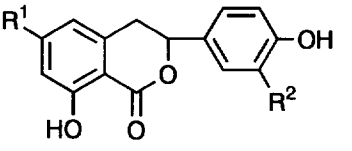
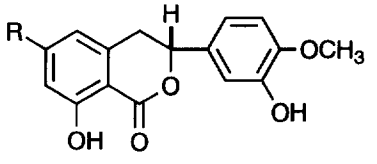


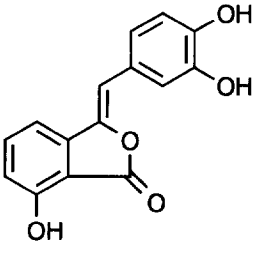
3' - deoxythunberginol A (**1**) : $R^1$=H, $R^2$=H
thunberginol A (**2**) : $R^1$=H, $R^2$=OH
thunberginol B (**3**) : $R^1$=OH, $R^2$=OH



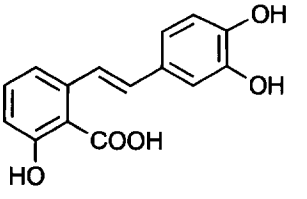
hydrangenol (**4**) : $R^1$=$R^2$=H
thunberginol G (**5**) : $R^1$=H, $R^2$=OH
thunberginol C (**6**) : $R^1$=OH, $R^2$=H
thunberginol D (**7**) : $R^1$= $R^2$=OH



phyllodulcin (**8**) : R = H
thunberginol E (**9**) : R=OH



thunberginol F (**10**)



3' - hydroxyhydrangeaic acid (**11**)

Fig. 1. Chemical constituents of Hydrangeae Dulcis Folium and their derivatives

specific T lymphocyte formation or activation, we investigated its effects on lymphocyte proliferation induced by various mitogens. Moreover, to determine the structural requirements for its immunomodulatory activity, we tested several related compounds including synthetic derivatives 3'-deoxythunberginol A (**1**) and 3'-hydroxyhydrangeaic acid (**11**). In this paper, we report the immunomodulatory effects of **2** and the other constituents of Hydrangeae Dulcis Folium on spleen lymphocyte activation.

**Methods**

The immunomodulatory activities of compounds on spleen lymphocyte proliferation were determined by MTT [(3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H tetrazolium bromide] assay.[6] Spleen cell suspensions were prepared according to the previous report.[7] Briefly, female C57BL/$_6$ mice aged 6 to 8 weeks were sacrificed by cervical dislocation and the spleens were removed. Spleens were mashed and filtered through stainless steel mesh (200 mesh) in RPMI-1640 (Gibco) medium. Single-cell suspensions were washed once with medium and

resuspended at $5.56x10^6$ cells/mL [lipopolysaccharide (LPS; Difco Laboratories, B5), concanavalin A (Con A; Wako Pure Chemical Industries)] or $2.22x10^7$ cells/mL [phytohemagglutinin (PHA; Difco Laboratories, PHA-P)] in RPMI-1640 medium containing 10 % fetal calf serum (Gibco), 100 units of penicillin G, 100 μg/mL of streptomycin, and 1mM sodium pyruvate. Aliquots of 90 μL of the cell suspension were seeded in 96-well culture plates (Falcon). Ten-μL aliquots of samples diluted in medium after dissolution in DMSO and the same volume of mitogens(LPS 100, Con A 10, PHA 200 μg/mL) were added and cultured at 37℃ under a 5% $CO_2$ atmosphere for 3 days. MTT assay was performed using a commercial kit (Cell titer 96™ Non-radioactive cell proliferation assay, Promega) and formazan dye products were measured by absorbance at 562 nm (reference wave length 660 nm) using a microplate reader (Model EL340, Bio-Tek™ Instruments).

**Results and Discussion**

Figure 2 shows the effects of thunberginols (**1**, **2**, **3**, **5**, **6**, **7**, **9**, **10**), hydrangenol (**4**), phyllodulcin (**8**), and 3'-hydroxyhydrangeaic acid (**11**) on lymphocyte proliferation. Thunberginol A (**2**) significantly suppressed B lymphocyte proliferation induced by LPS at $10^{-5}$M. Hydrocortisone, which had been used as anti-inflammatory drug, similarly inhibited the proliferation at lower concentration ($10^{-7}$M). The potent immunosuppressant cyclosporin A, which mainly acts on helper T lymphocytes,[8] had no significant effect. The other thunberginols (**1**, **3**, **5**, **6**, **7**, **9**, **10**), hydrangenol (**4**), phyllodulcin (**8**), and 3'-hydroxyhydrangeaic acid (**11**) significantly potentiated B lymphocyte proliferation at $10^{-5}$M. On the other hand, **2** and **4** significantly suppressed T lymphocyte proliferation induced by Con A from $10^{-6}$ to $10^{-5}$M and $10^{-5}$M, respectively. Moreover, thunberginol B (**3**) also slighitly suppressed proliferation at $10^{-5}$M. However, no compounds from Hydrangeae Dulcis Folium showed a suppressive effect on T lymphocyte proliferation induced by PHA. Hydrocortisone and cyclosporin A showed potent suppressive effects on the proliferation caused by both Con A and PHA at low concentrations ($10^{-7}$M).

We previously reported that thunberginol A (**2**) suppressed DTH reaction caused by $T_{DTH}$ lymphocytes sensitized with thymus-dependent antigens such as dinitrofluorobenzene and SRBC, mainly in the induction phase.[4] The DTH reaction can be classified into induction and effector phases.[9] At the induction phase, the APC take antigen into the cell by phagocytosis or endocytosis, and present antigen fragments on their surface with class II major histocompatibility complex (MHC II). Subsequently, native T lymphocytes connect with the antigen-MHC II complex on the APC *via* their T cell receptors (TCR). Thus, T lymphocytes acquire antigen information and can react to specific antigens. In the following effector phase, $T_{DTH}$ lymphocytes are activated by specific antigens and release various cytokines such as MAF (macrophage migration activating factor) and MIF (macrophage migration inhibitory factor).[10] As a result, macrophages, which accumulate in the region, release lysozomal enzymes and cause inflammation. In these processes, **2** is thought to control T lymphocyte sensitization or activation, because the DTH reaction had been suppressed by oral administration of this agent prior to the DTH reaction. So, we examined the effect of **2** on the nonspecific lymphocyte proliferation activated by various mitogens. Thunberginol A (**2**) dose-dependently suppressed lymphocyte proliferation induced by Con A and LPS. Although the inhibitory mechanism of **2** has not been clarified, this agent seemed to possess higher specificity for lymphocytes than hydrocortisone as **2** had no influence on PHA proliferation, while

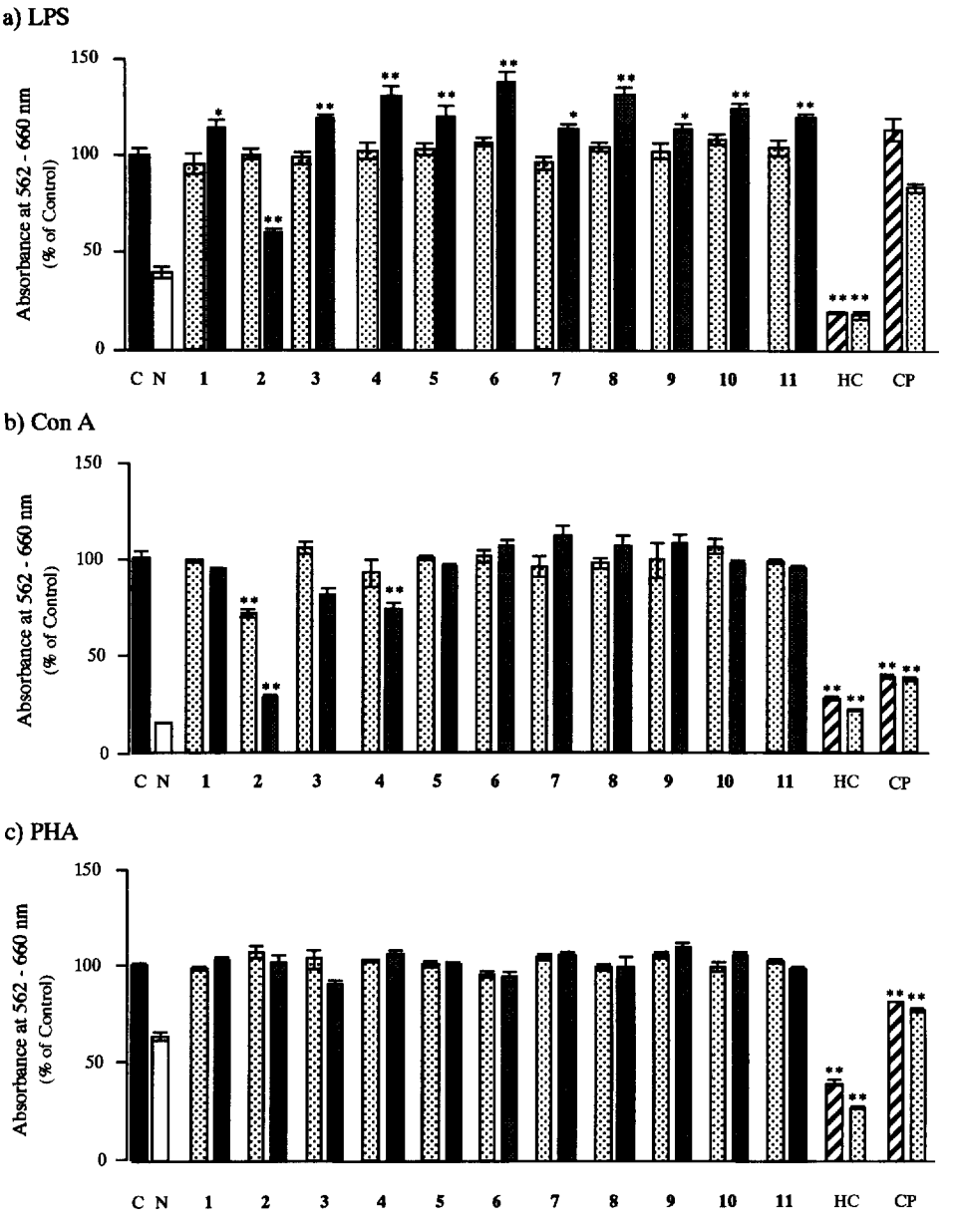



Fig. 2. Effects of constituents and their derivatives from Hydrangeae Dulcis Folium, hydrocortisone (HC) and cyclosporin A (CP) on lymphocyte proliferation activated by LPS (a), Con A (b) and PHA (c).
N, nonmitogen-stimulating group; C, control group. Each column represents the mean with S.E. of 4 to 6 experiments. Concentrations of samples : ▨, $10^{-7}$M; ▩, $10^{-6}$M; ■, $10^{-5}$M. Asterisks denote significant differences from the control group: **, $p<0.01$; *, $p<0.05$.

hydrocortisone suppressed all mitogen-induced proliferation. Moreover, the mechanism of suppression and targeting of the T lymphocyte subclass of **2** seemed to differ from those of cyclosporin A[11] as **2** inhibited only the Con A-induced activation, while cyclosporin A inhibited both Con A- and PHA- induced activation. Thus, it was suggested that the antigen-nonspecific suppressive effect of **2** for T lymphocytes contributed to DTH inhibition. In addition, we examined the actions of the other constituents of Hydrangeae Dulcis Folium and related compounds. Hydrangenol (**4**) and thunberginol B (**3**) weakly suppressed Con A-induced proliferation at $10^{-5}$M. We next examined the structural differences between active compounds **2**, **3**, and **4** and the inactive compound **11**. An isocoumarin moiety which possesses a lactone ring was suggested to be necessary for T lymphocyte suppression. Moreover, on examination of 3-phenyl isocoumarins (**1**, **2**, **3**) which possess a 8-OH group, **1** lacking the 3'-OH group had no effect and **3** with the 6-OH group had the less activity than **2**. These results suggested that the 3'-OH group of 3-phenyl isocoumarin moiety is necessary for T lymphocyte suppression, and the 6-OH group in the isocoumarin moiety reduced the activty. On the other hand, on examination of 3-phenyl dihydroisocoumarins (**4**, **5**, **6**, **7**) which possess the 8-OH group, **5**, **6**, and **7** which possess the 6-OH or the 3'-OH group had no effect. Thus, it seemed that the 6-OH group was not necessary for T lymphocyte suppression of 3-phenyl dihydroisocoumarins as well as 3-phenyl isocoumarin. Thus, the 3'-OH group also seemed to not be necessary for the activity in contrast to the case with 3-phenyl isocoumarins. On the other hand, all compounds from Hydrangeae Dulcis Folium except **2** significantly increased B lymphocyte proliferation induced by LPS at $10^{-5}$M. The structural requirements for this effect on B lymphocyte proliferation and distinction between **2** and the other constituents have not yet been clarified. However, it was interesting that these constituents showed mitogenic activity for B lymphocytes. In conclusion, thunberginol A (**2**) suppressed B and T lymphocytes proliferation induced by LPS and concanavalin A, therefore these inhibitory effects of **2** seemed to contribute to the suppressive effect on type IV allergy.

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