Pharmacological Effects of Urinary Products Obtained after Treatment with Saiboku-To, a Herbal Medicine for Bronchial Asthma, on Type IV Allergic Reaction

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Abstract: **To define the anti-allergic components in Saiboku-To, a herbal medicine for bronchial asthma, we examined the effects of 11 compounds found in post-administrative urine of Saiboku-To on concanavalin A-induced human lymphocyte blastogenesis *in vitro* and picryl chloride (PC)-induced mouse ear swelling *in vivo*. The urinary products of Saiboku-To were flavonoids and lignans derived from the constitutional herbs and their hydrogenated metabolites. Medicarpin derived from *Glycyrrhiza glabra*, magnolol and 8,9-dihydroxydihydromagnolol from *Magnolia officinalis*, baicalein, wogonin and oroxylin A from *Suctellaria baicalensis* inhibited lymphocyte blastogenesis in dose-dependent fashion with IC_{50} values ranging from 3.0 to 7.7 μg/mL, which corresponded to 20 – 100 times that of prednisolone IC_{50} (0.08 μg/mL). Davidigenin, dihydrowogonin and dihydrooroxylin A, which are hydrogenated metabolites of liquiritigenin, wogonin and oroxylin A, respectively, had no or little effects on lymphocyte blastogenesis. Oral administration of Saiboku-To, medicarpin, baicalein, magnolol and baicalin (100 mg/kg), inhibited PC-induced ear swelling significantly by 23.5, 40.1, 30.5, 23.6 and 20.9%, respectively, though the effects were weaker than that of 5 mg/kg of prednisolone (52.9%). The results suggested that flavonoids and lignans tested in the present study were implicated in anti-asthmatic effect of Saiboku-To through suppression of type IV allergic reaction.**

Key words: **Saiboku-To, *Magnolia officinalis*, *Suctellaria baicalensis*, *Glycyrrhiza glabra*, urinary products, type IV allergic reactions, lymphocyte blastogenesis, picryl chloride-induced mouse ear swelling.**

Introduction

Bronchial asthma has been widely recognized as a chronic inflammatory disorder of the airways. Airway inflammation involves activation of many types of cells such as lymphocytes, mast cells and eosinophils. The participation of these cells in the inflammation causes airways to be hyper-responsive. In particular, T lymphocytes play important roles in developing allergic inflammation by helping IgE production and stimulating eosinophils at the airway endothelium. Suppression of T lymphocyte action is, therefore, an effective aim in the relief of asthma symptoms (1), (2). Glucocorticoids (GC), strong anti-inflammatory drugs, are influencing both mediator release from peripheral blood mononuclear cells (PBMCs) (3) and T lymphocyte activation (4) and are used for severe bronchial asthma. However, it is desirable to reduce the GC dose without exacerbation of the asthma symptoms, thus minimizing the side effects.

Saiboku-To, a Kampo medicine (traditional Chinese remedy) comprising ten kinds of herbal extracts, has been used in Japan for GC-dependent asthmatic patients with the aim of reducing GC dose (5). The anti-allergic action of Saiboku-To based on the suppression of type I and IV allergic reaction has been confirmed in animal experiments of passive cutaneous anaphylaxis and picryl chloride (PC)-induced contact dermatitis (6). In addition, Saiboku-To enhances serum prednisolone concentrations by inhibiting a metabolic enzyme, 11β-hydroxysteroid dehydrogenase (7). These pharmacological activities may lead to GC sparing in the treatment of bronchial asthma; however, the active ingredients have not been clarified yet.

As the active candidates, we found 8 phenolic compounds (lignans and flavonoids) in the urine of patients receiving Saiboku-To (8). Recently, we further identified the metabolites in the urine, which were produced by bacterial hydrogenation in the intestine (9). In the present study, to confirm the anti-allergic activities of the phenolic compounds (Fig. **1**), we carried out *in vitro* and *in vivo* experiments in respect to type IV allergic reactions, focusing on concanavalin A induced human lymphocyte blastogenesis and the PC-induced contact dermatitis in mice, respectively.

Materials and Methods

Materials

Saiboku-To (TJ-96, Tsumura Co., Tokyo, Japan) used in this study is a granule formulation. Magnolol, baicalin, baicalein, and wogonin were purchased from Wako Chemicals (Osaka, Japan). Oroxylin A was contributed by Tsumura Co. Medicarpin was synthesized in our laboratory according to the method of Miller et al. (10). 8,9-Dihydroxydihydromagnolol was prepared by us from magnolol by osmic acid oxidation (11). Liquiritigenin was isolated from *Glycyrrhiza glabra* according to Shibata and Saitoh (12). Davidigenin was synthesized by

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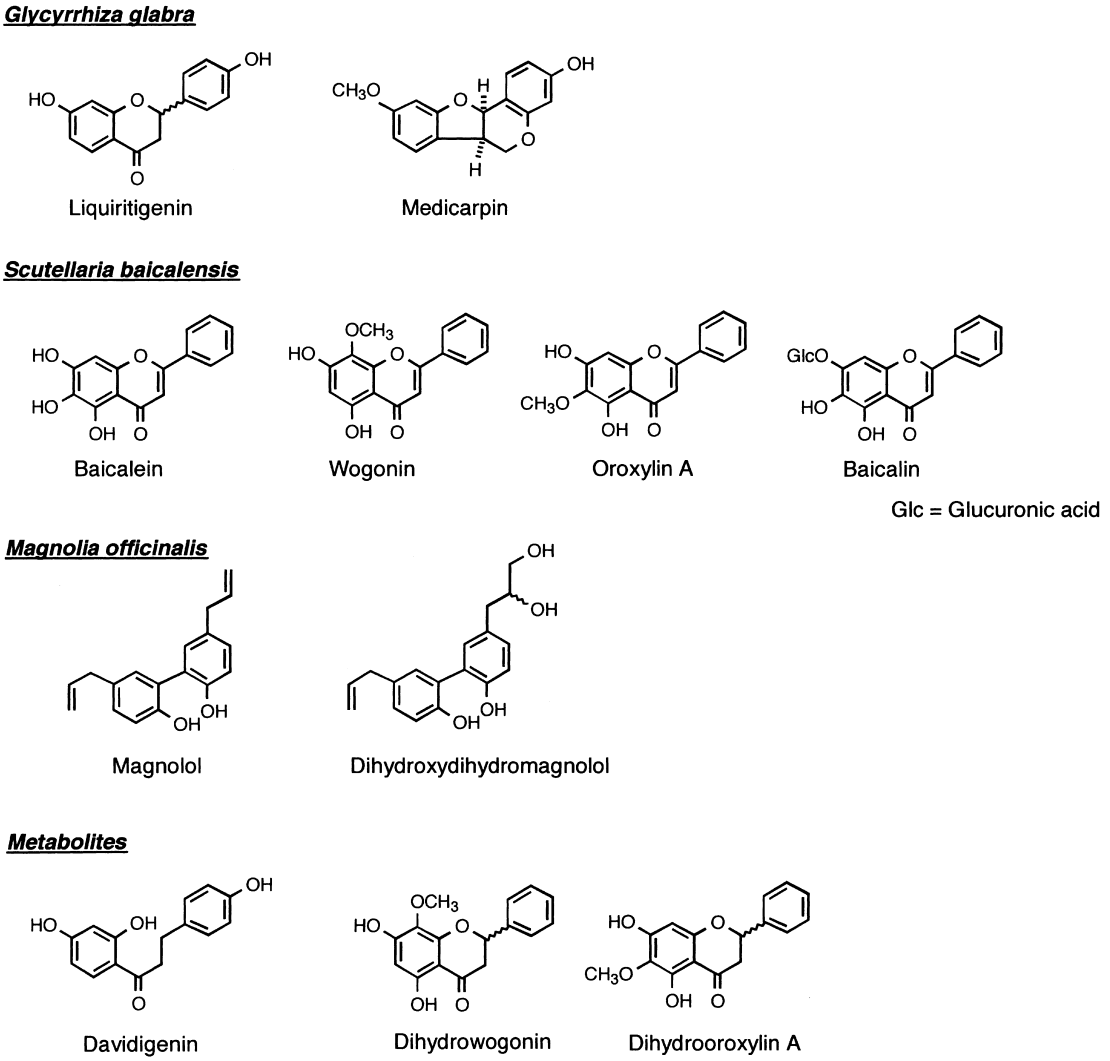

Fig. 1 Chemical structure of urinary products of Saiboku-To. Liquiritigenin, medicarpin, baicalein, wogonin, oroxylin A, baicalin, magnolol and dihydroxydihydromagnolol were found in both constitutional herbal extracts and post-administrative urine of Saiboku-To. The metabolites, davidigenin, dihydrowogonin and dihydrooroxylin were found only in post-administrative urine of Saiboku-To.

hydrogenation of isoliquiritigenin, which was prepared by aldol condensation of 2′,4′-dihydroxyacetophenone and *p*-hydroxybenzaldehyde according to the method of Miura et al. (13). Dihydrowogonin and dihydrooroxylin A were isolated in pure form from the urine samples collected from a subject receiving Saiboku-To by HPLC (9). All test compounds gave a single peak in HPLC analysis. Prednisolone was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Lymphocyte culture

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized venous blood collected from 12 healthy volunteers (9 males and 3 females), as described previously (14). The PBMCs were suspended in 10% fetal bovine serum (Bio-Cell, CA, USA) containing RPMI-1640 medium (Gibco BRL, NY, USA) to prepare a cell density of 1×10^6 cells/mL. Two hundred microliters of this suspension were placed into each well of 96-well flat-bottomed plates. Concanavalin A (Seikagaku Kogyo, Tokyo, Japan), a T-cell mitogen, was added to each well at a concentration of 5 μg/mL. Subsequently, each test compound or prednisolone dissolved in ethanol was added at the final concentrations of 0.01, 0.1, 1.0, or 10 μg/mL. After incubating for 4 days in 5% CO_2/air at 37 °C, the cells were pulsed with 18.5 kBq/well of [^3H]thymidine (New England Nuclear Co., USA) and were further incubated for 16 h. The cells were collected on glass-fiber filter paper using a multiharvester device and the radioactivity retained on the fiber was measured by a liquid scintillation counter. The mean of the counts of triplicate measurement for each test sample was determined. The concentration giving 50% inhibition of [^3H]thymidine incorporation (IC_{50}) was determined from the dose-response curve. The influence of the test compounds at the concentration of 10 μg/mL on cell viability was determined by a trypan blue dye exclusion test.

Ear swelling assay

Mouse ear swelling assay was conducted according to the method of Asherson and Ptak (15). Male ddY mice (Tokyo Experimental Animal Co., Tokyo, Japan), 7 weeks old, were used throughout the experiment. They were housed in our animal facilities and provided food and water ad libitum. After shaving the ventral surface, 0.1 mL of 7% PC (Nacalai Tesque, Inc., Kyoto, Japan) dissolved in ethanol was applied to the surface. On day 6 after induction with PC, mice were challenged by applying 0.02 mL of 1% PC dissolved in an olive oil on each side of the ear. Suspension of the test compounds and Saiboku-To granules in 1.5% gum Arabic (Sigma Chemical Co., St. Louis, MO, USA) aqueous solution were orally administered to the animals at the dose of 100 mg/kg, just before and 16 h after the PC challenge. Prednisolone as a positive control was administered at the dose of 5 mg/kg. Ear thickness was measured with dial thickness gauge (Ozaki MFG. Co. LTD., Tokyo, japan) at 24h after the challenge. Percent swelling was calculated according to the following formula:

$$\% \text{ Swelling} = \frac{\begin{array}{c}(\text{Thickness of the ear after challenge}) - \\ (\text{Thickness of the ear before challenge})\end{array}}{(\text{Thickness of the ear before challenge})} \times 100$$

Statistics

Statistical significance of the data was examined by Mann-Whitney's U-tests. The p values less than 0.05 were considered to be significant.

Results

Effects of phenolic compounds and prednisolone on lymphocyte blastogenesis

Dose-response curves of phenolic compounds and prednisolone on concanavalin A-induced blastogenesis of PBMCs from 4 – 12 healthy subjects were shown in Figure **2**. Medicarpin derived from *G. glabra*, magnolol and 8,9-dihydroxydihydromagnolol from *M. officinalis*, baicalein, wogonin and oroxylin A from *S. bicalensis* suppressed the blastogenesis dose-dependently. Their IC_{50} values ranged from 3.0 to 7.7 μg/mL, which were 40 – 100 times greater than that of prednisolone (IC_{50} = 0.08 μg/mL). Liquiritigenin from *G. glabra*, and baicalin (baicalein glucuronide) from *S. bicalensis*, exhibited 10 – 40% inhibition at the concentration of 10 μg/mL in several PBMCs. Davidigenin, dihydrowogonin and dihydrooroxylin A, had no effect on concanavalin A-induced T lymphocyte proliferation ($IC_{50} \geq 10\,\mu$g/mL). The results were summarized in Table **1**.

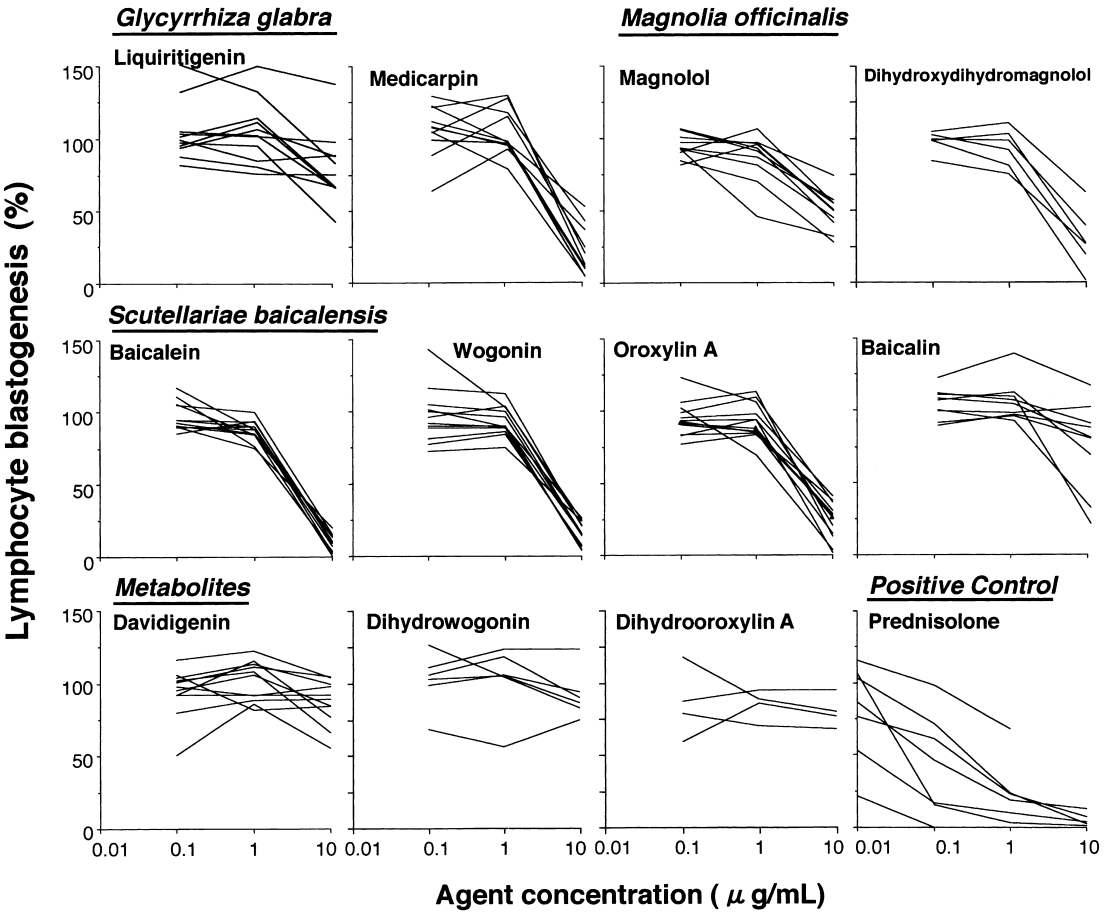

Fig. 2 Effects of urinary products of Saiboku-To and prednisolone on concanavalin A stimulated PBMCs obtained from healthy subjects (n = 4 – 12).

Cell viability examined by a trypan blue dye exclusion test at a concentration of 10 μg/mL was more than 92% for all compounds, suggesting that the influence of the test compounds on PBMCs was not caused by cytotoxicity.

Effects of phenolic compounds, Saiboku-To and prednisolone on ear swelling in PC-induced dermatitis

Control mice treated by the vehicle alone exhibited erythema and edema on an ear 24 h after challenge. The ear thickness in PC-sensitized mice was significantly increased by the 2nd PC challenge compared with that in non PC-sensitized mice (53.2 ± 3.1 vs. 38.0 ± 1.8 mm × 10^{-2}, $p < 0.001$). Pre-treatment of the test compounds or Saiboku-To reduced the ear thickness in the PC-sensitized mice. The percent inhibition of ear swelling for seven phenolic compounds, Saiboku-To and prednisolone are compared in Figure **3**. Prednisolone (5 mg/kg), a positive control, inhibited ear swelling by 52.9%. Saiboku-To (100 mg/kg) produced 23.5% inhibition ($p < 0.05$), which was almost equivalent to previous reports (6). Medicarpin, baicalein, magnolol and baicalin (100 mg/kg) significantly inhibited ear swelling by 40.1, 30.5, 23.6, and 20.9%, respectively ($p > 0.05$). Other phenolic compounds, wogonin, liquiritigenin and davidigenin were less effective.

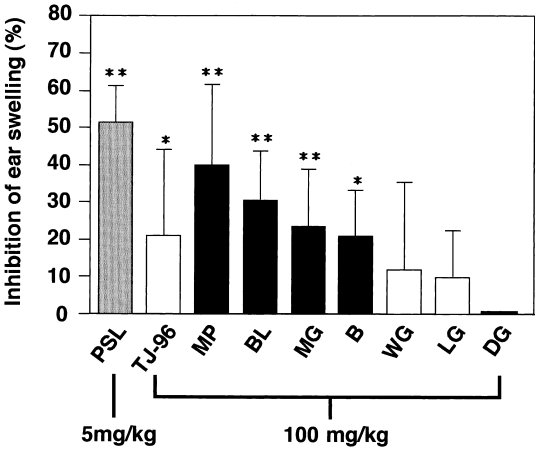

Fig. 3 Inhibitory effect of Saiboku-To, urinary products of Saiboku-To and prednisolone on ear swelling in PC-induced dermatitis mice. PSL: Prednisolone, TJ-96: Saiboku-To, MP: Medicarpin, BL: Baicalein, MG: Magnolol, B: Baicalin, WG: Wogonin, LG: Liquiritigenin, DG: Davidigenin. The bar of each group indicates mean ± SD of seven mice. Statistical significance based on the control group: $^{*}p < 0.05$; $^{**}p < 0.01$.

Discussion

Pharmacokinetic study of herbal medicine is an important approach to identify the active ingredients and to clarify how a medicine works on a particular disease. The phenolic compounds, including human metabolites, tested in the present study are actually absorbed into the body and achieved pharmacokinetically appropriate urine concentration after administration of Saiboku-To (8). Our interest, therefore, was focused on how these compounds contribute to anti-asthmatic effects of Saiboku-To, such as inhibition of type IV allergic reaction.

It is generally accepted that concanavalin A selectively stimulates human T cells (16) and that activated T cells play an important role for type IV allergic reaction (6). Six phenolic compounds showed apparent inhibitory effects on concanavalin A-induced lymphocytes proliferation. The most effective compound being baicalein from *S. baicalensis*. Other flavonoids from *S. baicalensis*, wogonin and oroxylin A, also showed an inhibitory effect with similar IC_{50} values compared to baicalein. However, dihydrowogonin and dihydrooroxylin A, hydrogenated metabolites of wogonin and oroxylin A, respectively, had no effects on lymphocyte proliferation. This observation suggests that hydrogenation was a deactivation process for wogonin and oroxylin A in terms of anti-lymphocyte activity. Medicarpin from *G. glabra*, dihydroxydihydromagnolol and magnolol from *M. officinalis*, also showed significant suppression on lymphocyte proliferation, though the activities were weaker than flavonoids from *S. baicalensis*. Anti-lymphocyte activity of medicarpin and dihydroxydihydromagnolol was confirmed for the first time in the present investigation. Magnolol and dihydroxydihydromagnolol, which is partly produced from magnolol in the body (11), showed almost equal anti-lymphocyte activity. These results suggested that several urinary products of Saiboku-To possess similar activity with prednisolone in terms of suppression of activated T-cells.

Table 1 Effects of urinary products of Saiboku-To and prednisolone on lymphocyte blastogenesis *in vitro*.

Compound	n	IC_{50} μg/mL median	range
Liquiritigenin	11	>10	6.5 ~ >10
Medicarpin	12	3.3	2.3 ~ >10
Magnolol	10	7.7	0.8 ~ >10
Dihydoxydihydromagnolol	6	4.3	2.5 ~ >10
Baicalein	12	3.0	2.3 ~ 4.1
Wogonin	12	3.7	2.8 ~ 4.8
Oroxylin A	12	4.3	2.0 ~ 6.5
Baicalin	10	>10	4.4 ~ >10
Davidigenin	11	>10	–
Dihydrowogonin	6	>10	–
Dihydooroxylin A	4	>10	–
Prednisolone	7	0.08	<0.01 ~ >1.0

We further examined the effects of seven phenolic compounds on type IV allergic reaction by using the ear swelling test on PC-sensitized mice. Each compound was used at the dose of 100 mg/kg, which corresponded to 20 times that of prednisolone (5 mg/kg), because the inhibitory effects of phenolic compounds on PBMC proliferation *in vitro* were 20 – 100 times weaker than prednisolone. In agreement with previous reports, PC-induced ear swelling was suppressed by oral administration of Saiboku-To (6). The phenolic compounds, baicalein, magnolol, and medicarpin, which showed inhibitory effects on lymphocyte proliferation *in vitro*, suppressed the ear swelling significantly. Wogonin also showed suppressive activity on ear swelling, though the effect was not significant. On the other hand, liquiritigenin and davidigenin, which did not show any effect *in vitro*, were also inactive in the PC-induced ear swelling. These observations both *in vitro* and *in vivo* suggested that the effects of the phenolic compounds on

PC-induced ear swelling, at least partly, resulted from suppression of T lymphocyte activation. Baicalin (baicalein glucuronide) was ruled out, because it showed significant effects on the ear swelling even though the inhibitory effects on lymphocyte proliferation were weak. Baicalin may be hydrolyzed to active baicalein by intestinal bacteria enzymes (17), which strongly inhibits both PBMC proliferation and PC-induced ear swelling.

Since the number of eosinophils has been known to increase in the inflammatory site at 24 h after PC challenge (18) in this animal model, it is reasonable to consider that the phenolic compounds tested in the present study suppress the ear swelling by inhibition of the eosinophilia, releasing strong bronchoconstrictors, cysteinyl-containing leukotriene (LT) and platelet-activating factor. In the previous study, we observed that baicalein, medicarpin and magnolol strongly inhibited LTB_4 and LTC_4 release from human PBMCs stimulated with calcium ionophore A23187 (19). Taken together, it is suggested that baicalein, medicarpin and magnolol are implicated in the suppressive effect of Saiboku-To on type IV allergic ear swelling through inhibition of both lymphocyte proliferation and chemical mediators released from PBMCs.

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References

[1] Broide DH, Gleich GJ, Cuomo AJ, Coburm DA, Federman EC, Schwartz LB, Wasserman SI. Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. J. Allergy Clin. Immunol. 1991; 88: 637 – 48

[2] Bousquet J, Chanez P, Lascoste JY, Barneon G, Ghavanian N, Enander I, Veng P, Ahlstedt S, Simony-Lafontaine J, Godard P. Eosinophilic inflammation in asthma. N. Engl. J.Med. 1990; 323: 1033 – 9

[3] Vecchiarelli A, Siracusa A, Cenci E, Puliti M, Abbritti G. Effect of corticosteroid treatment on interleukin-1 and tumour necrosis factor secretion by monocytes from subjects with asthma. Clin. Exp. Allergy 1992; 22: 365 – 70

[4] Hirano T, Homma M, Oka K, Tsushima H, Niitsuma T, Hayashi T. Individual variations in lymphocyte-responses to glucocorticoids in patients with bronchial asthma: comparison of potencies for five glucocorticoids. Immunopharmacology 1998; 40: 57 – 66

[5] Nagano H, Kobayashi S, Nakajima S, Egashira Y. Long-term clinical evaluation of Saiboku-To, an anti-asthmatic agent, in treatment of bronchial asthma (Multicenter Open Trial). Respiration 1988; 7: 76 – 87 (in Japanese)

[6] Koda A, Nishiyori T, Nagai H, Matsuura N, Tsuchiya H. Anti-allergic actions of crude drugs and blended Chinese traditional medicines. Effects on type I and IV allergic reactions. Folia Pharmacol. Jpn. 1982; 80: 31 – 41

[7] Homma M, Oka K, Ikeshima K, Takahashi N, Niitsuma T, Fukuda T, Hayashi T. Different effects of traditional Chinese medicines containing similar herbal constituents on prednisolone pharmacokinetics. J. Pharm. Pharmacol. 1995; 47: 687 – 92

[8] Homma M, Oka K, Taniguchi C, Niitsuma T, Hayashi T. Systematic analysis of post-administrative Saiboku-To urine by liquid chromatography to determine pharmacokinetics of traditional Chinese medicine. Biomed. Chromatogr. 1997; 11: 125 – 31

[9] Li C, Homma M, Ohkura N, Oka K. Stereochemistry and putative origins of flavanones found in post-administration urine of traditional Chinese remedies Shosaiko-to and Daisaiko-to. Chem. Pharm. Bull. 1998; 46: 807 – 11

[10] Miller DK, Sadowski S, Han GQ, Joshua H. Identification and isolation of medicarpin and a substituted benzofuran as potent leukotriene inhibitors in an anti-inflammatory Chinese herb. Prostag. Leukotr. Essent. Fatty Acids 1989; 38: 137 – 43

[11] Homma M, Oka K, Yamada T, Niitsuma T, Itoh H, Takahashi N. A strategy for discovering biologically active compounds with high probability in traditional Chinese herb remedies: An application of Saiboku-To in bronchial asthma. Anal. Biochem. 1992; 202: 179 – 87

[12] Shibata S, Saitoh T. Flavonoid compounds in licorice root. J. Ind. Chem. Soc. 1978; 55: 1184 – 91

[13] Miura H, Kitamura Y, Sugii M. Determination and synthesis of confusoside, a minor dihydrochalcone glycoside from *Symplocos microcalyx* Hay. Nat. Med. 1985; 39: 312 – 5

[14] Hirano T, Oka K, Kawashima E, Akiba M. Effects of synthetic and naturally occurring flavonoids on mitogen-induced proliferation of human peripheral-blood lymphocytes. Life Sci. 1989; 45: 1407 – 11

[15] Asherson GL, Ptak W. Contact and delayed hypersensitivity in the mouse. I. Active sensitization and passive transfer. Immunology 1968; 15: 405 – 16

[16] Greaves MF, Janossy G, Doenhoff M. Activation of human T and B lymphocytes by polyclonal mitogens. Nature 1974; 248: 698 – 701

[17] Kim DH, Jung EA, Sohng IS, Han JA, Kim TH, Han MJ. Intestinal bacterial metabolism of flavonoids and its relation to some biological activities. Arch. Pharm. Res. 1998; 21: 17 – 23

[18] Lavaud P, Rodrigue F, Carré C, Touvay C, Mencia-Huerta JM, Braquet P. Pharmacologic modulation of picryl chloride-induced contact dermatitis in the mouse. J. Invest. Dermatol. 1991; 91: 101 – 5

[19] Homma M, Taniguchi C, Minami M, Oka K, Yamamoto S, Niitsuma T, Hayashi T. Discovery of biologically active compounds from human administered Kampo-medicine XVI. Pharmacological activities of urinary products of Saiboku-To. J. Med. Pharm. Soc. Wakanyaku 1995; 12: 448 – 9 (in Japanese)

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