

A Proposal of Dietary Supplement from Choto-san, a Kampo Medicine

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(Received 31 March)

Abstract – Therapeutic effect of a Kampo medicine, Choto-san, in patients with vascular dementia was demonstrated by a double-blind and placebo-controlled clinical trial. To clarify the therapeutic efficacy of Choto-san, anti-ischemic effect in mice, hypotensive effect in spontaneously hypertensive rats (SHR), anti-oxidative effects *in vitro*, and N-methyl-D-aspartate (NMDA) receptor-blocking activity using *Xenopus* oocytes were studied. (1) Pretreatment with Choto-san (0.75 - 6.0 g/kg, p.o.) or a component herb Chotoko (*Uncaria* genus: 75 - 600 mg/kg, p.o.) prevented ischemia-induced impairment of spatial learning behaviour in mice. Indole alkaloids- and phenolic fractions extracted from Chotoko also improved significantly the learning deficit. (2) Subchronic administration of Choto-san (0.5 g/kg, p.o.) caused a significant hypotensive effects in SHR. (3) Choto-san, Chotoko, and the phenolic constituent, (-) epicatechin, significantly protected the NG108-15 cell injury induced by H₂O₂ exposure *in vitro* and also inhibited lipid peroxidation in the brain homogenate. (4) Indole alkaloids, rhynchophylline and isorhynchophylline (1-100 μ M), reversibly reduced NMDA-induced current in the receptor-expressed *Xenopus* oocytes. These results suggest that anti-vascular dementia effects of Choto-san are mainly due to the effect of Chotoko. From these results, it is possible to make a novel dietary supplement through several extraction steps from Chotoko.

Keywords □ Choto-san, *Uncaria* genus, indole alkaloids, rhynchophylline, isorhynchophylline, phenolic compounds

INTRODUCTION

Herbal medicines were first introduced to Japan by a Chinese monk "Ganjin wajo" in 5 AC. Since then, various Chinese herbal medicines together with the therapeutic methodology, which had been developed in China, were transmitted to Japan for over 1,000 years. Thereafter, traditional Chinese medicine developed as the Kampo medicine from the middle of 16th to the middle of 19th century in Japan.

Choto-san (Gouteng-san in Chinese) is one of the prescriptions in Kampo medicine, which consists of ten medicinal herbs and gypsum fibrosum (Table I). The original indication of Choto-san is for chronic headache and hypertension. Recently, therapeutic effect of Choto-san on vascular dementia was evaluated by the double blind and placebo controlled study. Choto-san (4.5 g/day, p.o.) and a placebo were each given three times a day to a group of patients with vascular dementia for 12 weeks. Choto-san was statistically superior to the placebo in

global improvement rating (GIR), utility rating, GIR of subjective symptoms (heaviness of head, headache, dizziness of vertigo, etc.), GIR of psychiatric symptoms (spontaneity, emotion, intellectual ability, etc.) and GIR of disturbance in daily living activities (sitting, standing, walking, washing face and hands, etc.) at the end of 12 weeks administration (Terasawa *et al.*, 1997).

To clarify the therapeutic efficacy of Choto-san, anti-ischemic effect in mice, hypotensive effect in spontaneously hypertensive rats (SHR), anti-oxidative effects *in vitro*, and N-methyl-D-aspartate (NMDA) receptor-blocking activity using *Xenopus* oocyte membrane were studied.

MATERIALS AND METHODS

Subjects

Male ICR mice at 8 weeks old and spontaneously hypertensive rats (SHR), SHR with stroke prone (SHR-SP) at 4 weeks old and age-matched Wistar-Kyoto rats were used in the present experiment. They were obtained from the colony of specific pathogen free mice and rats maintained by Japan SLC (Shizuoka, Japan). Mice and rats were housed in groups of 8

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Table I. Choto-san: Composition, Kampo Diagnosis, Indication and Usage

Item	Component herb	Constituents (Indicator)
Choto-san	Hook of <i>Uncaria sinensis</i> (Oliv.) Havi(3)	<i>Uncaria rhychophylla</i>
	Peel of <i>Citrus nushiu</i> Mare. (3)	idole alkaloids, polyphenols, tannis
	Tuber of <i>Pinellia ternata</i> Breit. (3)	essential oils
	Root of <i>Ophiopogon japonicus</i> Ker-Gawler (3)	homogentisic acid
	<i>Poria cocos</i> (Fr.) Wolff (3)	ophiopogonin A,B
	Root of <i>Panax ginseng</i> C.A.Mayer(2)	pachyman
	Flower of <i>Chrysanthemum morifolium</i> Hemsl. (2)	ginsenosides
	Root of <i>Ledebouriella seseloides</i> Woll. (2)	psolaren, deltoin
	Gypsum $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (5)	calcium
	Root of <i>Glycyrrhiza uralensis</i> (1)	glycyrrhizin
Tuber of <i>Zingiber officinale</i> Roscoe (1)	zingiberene	
Kampo diagnosis	Yang-disease 2 stage, Yang-deficiency, Oketsu type, KI-deficiency	
Indication	Chronic headache and hypertension	
Usage	4,5 g/day, p.o. (divided into two to three times)	

Note: Numeral in the parentheses indicates the weight of dried herbs (gram) which is decocted with boiled water every day. Patients drink the decoction three times a day before the meal.

per cage (31×20×13 cm) and in groups of 4 per cage (36×30×17 cm), respectively, on a 12 hr light/dark cycle (light on 07:30-19:30) at 24±1°C with constant humidity for at least 1 week before the experiment. Food and water were given ad libitum. All experiments were performed at the same time of day to avoid the effect of physiological cycling. All experimental procedures were performed in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals at Toyama Medical and Pharmaceutical University.

Drugs

Following drugs were purchased from indicated sources: (-)-epicatechin and caffeic acid from Sigma Chemical Co. (St. Louis, MO, USA); curcumin and tacrine from Nacalai Tesque, Inc. (Kyoto, Japan); H₂O₂ from Santoku Chemical Industries Co. (Tokyo, Japan); nifedipine from Bayer Japan (Osaka) and nicardipine hydrochloride from Nihon Iyakuin Kogyo (Toyama). Other reagents were purchased of the first grade from above companies.

Choto-san preparation

Twenty five grams of ten dried herbs (purchased from Tochimoto Co. Ltd., Osaka, Japan) were mixed, soaked in 300 ml of distilled water and decocted for 45 min at 100°C. Then three grams of *Uncaria sinensis* was added and decocted for 15 min more. The decoction was filtered, and the residue was decocted once more according to the same procedure. Finally, all filtered fractions were pooled and freeze-dried. The dried powder was

stored at room temperature in the desiccator.

Transient cerebral ischemia

Mice were subjected to transient cerebral ischemia following occlusion of the bilateral common carotid arteries one hour after the administration of the drug. In brief, the mouse was anesthetized with urethane (1.5 g/kg, i.p.). The bilateral common carotid arteries were exposed, carefully separated from the adjacent veins and sympathetic nerves, and occluded by artery clips (Roboz Surgical Instrument Co., Inc., MD, USA) for 20 min. During the arteries were clamped, 0.3 ml of the blood was withdrawn from the tail vein. Then, the artery clips were removed and cerebral blood flow was restored. The skin incision was closed and the mice were kept in an air-conditioned room at 25°C. Sham-operated mice were subjected to the same procedure without carotid clamping and withdrawal of the blood.

Morris water maze learning performance

A pool (70 cm diameter, with a depth of 13 cm of water maintained at 25±1°C) was placed in a dimly light and large test room and surrounded by visual cues. Before the start of learning, mice were given a pretraining session in which they were allowed to swim freely in the pool without an escape platform (5 cm diameter) and also with the platform located at 1 cm above the water level for 60 seconds. In the learning block, the platform was situated 1 cm below the surface of the water. The pool was divided into four quadrants with the platform in a

fixed position in one quadrant. Daily learning consisted of four trials in which the mouse was placed in the water from four different starting points and the latency of escaping onto the platform was recorded. This was conducted for 5 consecutive days. A maximum of 60 seconds was allowed during which the mouse had to find the platform and stayed on it. On the sixth day, each mouse was subjected to a probe trial without the platform, and the time of crossing the former platform quadrant and the total time of crossing all quadrants were recorded for 1 min.

Measurement of the blood pressure and heart rate in awake rats

Awake rats were lightly supported in a mesh holder made of cloth and the arterial blood pressure from the tail artery was indirectly measured using the tail-cuff apparatus (BP-98, Softron, Japan) equipped with a pair of photodiode and light emitting diode, an air pump and a pressure transducer. The tail-cuff was controlled with a personal computer. Signals from the photocell amplifier and cuff pressure transducer were recorded continuously on the monitor of the computer's display. Regression curve was calculated from peak values of each pulse volume oscillation by the method of least squares. Systolic and mean blood pressure were determined by the pressure at where this curve started and became maximum, respectively. Diastolic blood pressure was calculated from the values of systolic and mean blood pressure. All of these results were displayed and saved in the floppy disk.

Cell cultures and H₂O₂-induced oxidative cell damage in NG108-15 cells

NG108-15 cells were continuously cultured in Dulbecco's modified eagles's medium supplemented with 4% fetal bovine serum, 100 μ M hypoxanthine, 16 μ M thymidine, 1 μ M aminopterin and 1 μ g/ml minomycin. The culture medium was changed every 2-3 days. All cultures were maintained at 37°C under 10% CO₂ with 95% relative humidity. For experiments, cells were planted onto 3.5-cm polyornithine-coated plates and used after 3-4 days of incubation.

A test compound was first dissolved in 0.5% v/v dimethyl sulfoxide (DMSO) and later mixed with the culture medium to give a final concentration of 12.5 - 100 μ M. To evaluate the protective effect of the test compound on the cell damage induced by H₂O₂, the cells were incubated in culture medium containing 500 μ M H₂O₂ and 0.05% trypan blue with or without various concentrations of the test compound for 3 hrs. Then cell

viability was measured by the trypan blue exclusion method and expressed as the percentage of unstained cell among the total cells.

Malondialdehyde measurements and anti-lipid peroxidation activity

Malondialdehyde (MDA) was measured using a modified method (Esterbauer et al., 1991; Gluck et al., 2001). Brain tissue was homogenized in 1.15% KCl/0.4 mM of sodium azide and incubated at 37°C for 15 min. Proteins were precipitated by the addition of 20% (w/v) trichloroacetic acid. The tissue samples were centrifuged at 12,000 g for 15 min and the supernatant was added to an equal volume of 0.8% (w/v) 2-thiobarbituric acid. These samples were incubated at 100 for 15 min. After the cooling period, TBA-RS generated was spectrophotometrically determined at 532 nm. Bismalondialdehyde tetraethyl acetal was used as a standard. Protein concentrations were assayed by using Biuret method.

Isolation of total RNA, oocytes preparation and electrophysiological recordings

The cerebral cortex were dissected after the decapitation of male Wistar rats (12-14 weeks old) and frozen in liquid nitrogen. The frozen tissues were homogenized in Sepasol-RNA I Super (Nacalai Tesque, Kyoto) and the total RNA was extracted according to the protocol provided by the manufacturer. *Xenopus* oocytes at stage V or VI were prepared by the method described in the previous report (Leewanich *et al.*, 1998), injected with 47 nl of 5 mg/ml total RNA prepared from the cerebral cortex and incubated at 18°C for 2 days in modified Barth's solution supplemented with antibiotics. Oocytes expressing total RNA from the cerebral cortex were used to examine NMDA-, kainic acid- and AMPA-induced current responses. The membrane currents were recorded from oocytes at a holding potential of -60 mV using a two-electrode voltage clamp method (Gene Clamp 500, Axon instruments, Foster city, CA).

Data analysis

All results are expressed as the mean \pm S.E.M. Statistical significance between different groups for the Morris water maze test was analyzed by two-way analysis of variance (ANOVA) among the groups. A student's t-test was used for the analysis of significant differences between the two groups. One-way analysis of variance (ANOVA) followed by the Dunnett test was used for multiple comparisons. Differences of

$P < 0.05$ were considered significant.

RESULTS

Animal model for vascular dementia in mice: ischemia-induced impairment of spatial memory and effect of Choto-san

Mice exposed to transient cerebral ischemia were used as an animal model of vascular dementia and effects of Choto-san, the component herbs and the constituents on their impairment of spatial learning ability and memory were studied in water maze performance. The mice with the cerebral ischemia took a longer time to find the hidden platform than the sham-operated control during the learning trials in the water maze, although ischemia itself did not affect the swimming ability in the pre-training trial.

Pretreatment with Choto-san (750 - 6,000 mg/kg, p.o.) significantly prevented the ischemia-induced impairment of the spatial learning to find the platform in the water maze test (Fig. 1).

A component herb of Choto-san, Chotoko (75 - 600 mg/kg), an alkaloid fraction (188 mg/kg) and a phenolic fraction (188 mg/kg), or indole alkaloids rhynchophylline (10 mg/kg) and geissoschizine methylether (10 mg/kg), which were isolated from Chotoko, also inhibited the impairment of the learning ability in the training test for 5 days and significantly improved the memory in the probe test on the 6th day after the ischemia.

A reference agent, tacrine (1 and 2.5 mg/kg, i.p.), significantly inhibited the impairment of the learning ability in the water maze performance (data not shown).

Multi-action mechanisms (1): anti-hypertensive effects in SHR

To clarify action mechanisms of Choto-san, effects on hypertension in SHR and SHR-SP were studied. The subchronic administration of Choto-san at a dose of 0.5 g/kg/day, p.o., or Chotoko at a dose of 0.05 g/kg/day, p.o., produced a significant hypotensive effect in SHR and a tendency to inhibit the induction rate of the apoplexy in SHR-SP. While Saiko-keishi-to (0.5 and 5.0 g/kg/day, p.o.), another Kampo prescription of the negative control, affected neither the blood pressure nor the apoplexy. The subchronic administration of a reference agent, nicardipine, produced prominent hypotensive effect at doses 1 and 10 mg/kg/day, p.o., and inhibition of apoplexy at 10 mg/kg/day, p.o.

Multi-action mechanisms (2): an indirect mechanism -- anti-oxidant and anti-lipid peroxidation in vitro

To check other action mechanisms, anti-oxidative activity of Choto-san and the constituents was examined. Application of hydrogen peroxide to NG 108-15 cells *in vitro* significantly reduced the cell viability in a concentration-dependent manner with an IC_{50} of 500 μ M. Prior application of Choto-san (250 - 1,000 μ g/ml), Chotoko (250 - 1,000 μ g/ml) or the constituents of Choto-

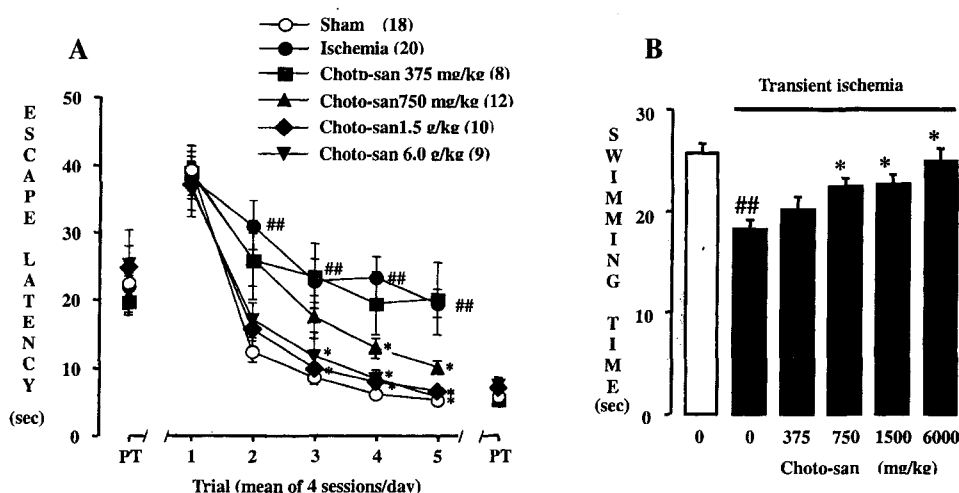


Fig. 1. Effect of Choto-san on transient ischemia (2VO)-induced impairment of Morris water maze performance in mice. Choto-san was orally given to animals one hour before the ischemia. Two days after the ischemia, the trial test was performed of 4 trials/block/day for 5 days. A: Time course of the change in the latency escaping to the platform in the pool. Each point represents the mean of the latency with the S.E.M. B: The swimming time in the platform quadrant was recorded in the platform trial for one minute after the platform was removed on 6th day of the test. Each column represents the mean swimming time in the quadrant with S.E.M. PT: platform is visible during the trial. ## $p < 0.01$, ### $p < 0.001$ vs. sham group. * $p < 0.05$, ** $p < 0.01$ vs. ischemic control. (cited from Watanabe et al.: Pharmacol. Biochem. Behav. **75**, 635, 2003)

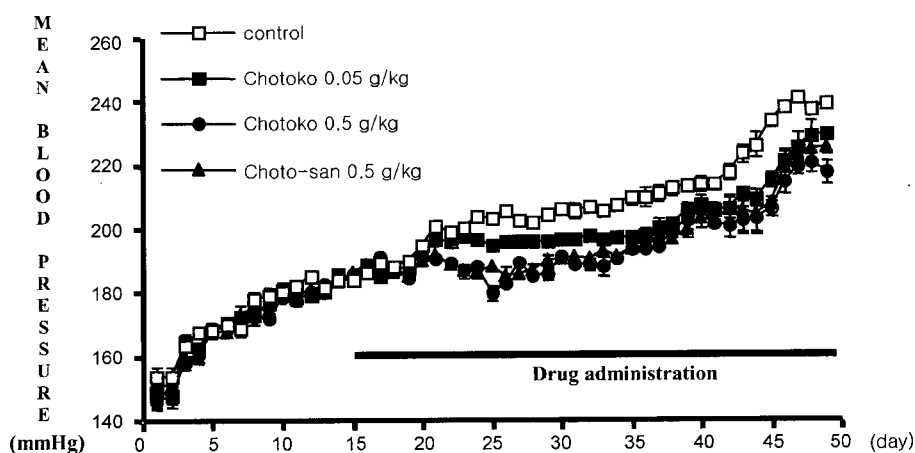


Fig. 2. Antihypertensive effect of subchronic administration of Choto-san extract in SHR-SP. Choto-san extract (0.5 g/kg/day), Chotoko (0.05 and 0.5 g/kg/day) or tap water (control) was given to animals as drinking water for 60 days after the blood pressure reached a level over 180 mmHg (n=8). The mean blood pressure significantly decreased at 10 days after the start of drinking of Choto-san.

ko, (-) epicatechin and caffeic acid (200 μ M), to 500 μ M of hydrogen peroxide significantly inhibited the reduction of the cell viability as compared to that of the control. A reference immunosuppressive ligand FK506 (100 - 1,000 nM) prominently inhibited the hydrogen peroxide-induced reduction of the cell viability in a concentration-dependent manner.

In the brain homogenate, Choto-san, methanol extract of Chotoko, (-)epicatechin and a reference agent of vitamin E showed a significant anti-lipid peroxidation activity with IC_{50} values of 124.7 μ g/ml, 4.6 μ g/ml, 39.3 μ M and 153.7 μ M, respectively.

Muti-action mechanisms (3): drug-receptor interactions -- Inhibition of NMDA receptor function with indole alkaloids

To examine a possible mechanism underlying actions of Choto-san and/or Choto-ko, effects of indole alkaloids isolated from *Uncaria rhynchophylla* on NMDA receptor function using a receptor expression model of *Xenopus* oocytes were investigated. Rhynchophylline and isorhynchophylline (1 - 100 μ M) per se did not affect membrane current, but reversibly reduced NMDA-induced current in a concentration-dependent but not voltage-dependent manner. The IC_{50} values of rhynchophylline and isorhynchophylline were of 43.2 and 48.3 μ M, respectively. While, those alkaloids produced no effect on the current mediated either by ionotropic kainic acid-type and (+)- α -3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors or by the metabotropic glutamate receptor 1 and 5. The alkaloids (30 μ M) significantly reduced the maximal current response evoked by NMDA and glycine (co-agonist of NMDA receptor), but had no effect on the EC_{50} values and Hill coefficient

of NMDA and glycine for inducing currents (data not shown).

DISCUSSION

Present result showed that Choto-san exerted protective effect on spatial learning impairment induced by transient cerebral ischemia in mice. The neuro-protective effect of Choto-san may be due at least partly to the anti-hypertensive, antioxidative and receptor blocking effects.

The decrease in cardiac norepinephrine content and the reductions in plasma angiotensin II, plasma aldosterone and urine levels of adrenaline and noradrenaline have been shown to be involved in the anti-hypertensive effect of Choto-san (Watanabe et al., 1987; Yokose et al., 2000). It has been reported that Choto-san also inhibits norepinephrine- and high K^+ -induced contractions in isolated mesenteric arteries in a concentration-dependent manner, suggesting that the anti-hypertensive effect of Choto-san is mediated by the calcium channel antagonistic action (Ishii et al., 1987). The calcium channel blocking activity of Choto-san has been demonstrated more clearly in the pharmacological analysis of hirsutine, one of indole alkaloids isolated from *Uncaria rhynchophylla* Miq (Horie et al., 1992; Watanabe et al., 1999; Yamahara et al., 1987; Yano et al., 1991). Furthermore, several alkaloids such as hirsutine, dihydrocorynantheine, geissoschizine methylether, rhynchophylline, and 3- α -dihydrocadambine extracted from *Uncaria rhynchophylla*, have been reported to induce hypotensive and vasodilative effects in anesthetized rats and dogs (Aisawa et al., 1985; Ozaki, 1989, 1990; Sakakibara, 1999). There is another possi-

bility that the anti-hypertensive effect of Choto-san is resulted from radical scavenging activity of Chotoko in the isolated aorta with the endothelium (Goto et al., 1998).

In *in vitro* study, Choto-san protected NG108-15 cells against hydrogen peroxide-induced damage in a concentration-dependent manner. Hydrogen peroxide is believed to cause cell damage by reacting with the cell membrane, resulting in lipid peroxidation of the membrane. Lipid peroxidation has been implicated as one of the main process responsible for ischemic cell damage. A number of studies have shown that pretreatment with either a free radical scavenger or antioxidants reduced ischemia reperfusion-induced lipid peroxidation. The inhibitory effect of Choto-san on lipid peroxidation may be contributed by its constituents. Choto-san was reported to contain many phenolic antioxidants such as epicatechin, caffeic acid, procyanidin and quercetin, which are known to prevent lipid peroxidation-induced cell damage by interrupting lipid peroxidation chain reactions initiated by free radicals at the cell membrane (Ishige et al., 2001; Tanaka et al., 2001). Chotoko has been reported to have antioxidant and free radical scavenging activities which are detected by electron spin resonance method and to inhibit lipid peroxidation in the brain of iron-induced epileptic rats (Liu and Mori, 1992)). Taken together with these results, present study suggests that Choto-san protects the brain due to the attenuation of free radicals formation following the ischemia.

NMDA subtype of glutamate receptor in the cerebral cortex and hippocampus plays an important role in learning and memory (Bliss and Collingridge, 1993; Cotman et al., 1989). While, excessive activation of NMDA receptors induces the neuronal cell death mediated by intracellular Ca^{2+} overload. Such excitotoxic neuronal death appears to contribute to various neurological disorders such as cerebrovascular dementia and Alzheimers disease (Cotman et al., 1989; Muller et al., 1995; Parsons et al., 1998). Various indole alkaloids isolated from *Uncaria sinensis* such as isorhynchophylline, isocorynoxine and rhychopylline at relatively high concentrations prevented the glutamate-induced cell death in cultured cerebellar granule cells and global cerebral ischemia in rats (Shimada et al., 1999; Suk et al., 2002). Furthermore, NMDA receptor antagonists provided the protection against neuronal damage attributed to cerebral ischemia (Block, 1999; de Zoppo et al., 1997). Present findings suggest a possibility that rhynchophylline exerts noncompetitive antagonism by allosterically inhibiting NMDA binding to its recognition site and/or glycine binding to the glycine recognition site on the NMDA receptor channel protein. However, we need a

further study to identify the site of action of rhynchophylline on the NMDA receptors.

Thus, present results suggest that the anti-dementia effect of Choto-san is total effects of component herbs, i.e. anti-hypertensive, free radical scavenging, and anti-excitotoxicity effects which are attributed partly to phenolic compounds and indole alkaloids contained in Chotoko.

A proposal of a new dietary supplement from Chotoko

Experimental and pharmacological profile of Choto-san is quite similar to that of a component herb Choto-ko. Thus, it is possible to prepare an alternative medicine or a dietary supplement from Choto-ko after eliminating unnecessary substances (biflavones, tannins, saponosides, coumarins) and concentrating active substances (flavone glycosides, terpene lactones, etc.) which are considered to be active in the clinical efficacy. The extraction procedure and the chemical definition of the extract should be controlled and standardized.

ACKNOWLEDGMENTS

FK506 was donated by Fujisawa Pharmaceutical Co. (Osaka, Japan). This work was supported by a Grant-in-Aid for Leading Research Utilizing Potential of Regional Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

This research work was done in collaboration with Drs. Tae-Hyun Kang, Qi Zhao, Kinzo Matsumoto, Michihisa Tohda, Yukihisa Murakami, Shao-Hui Zhang and Pramote Mahakunakorn (Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-0194, Japan), Iwao Sakakibara (Tsumura Central Research Laboratories, Ibaraki 300-1192, Japan), Norio Aimi and Hiromitsu Takayama (Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan).

This research was supported in part by a 21st Century COE Program.

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