The Screening of Chinese Crude Drugs for Ca^{2+} Antagonist Activity: Identification of Active Principles from the Aerial Part of *Pogostemon cablin* and the Fruits of *Prunus mume*[1)]

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Hot aqueous extracts of 134 Chinese crude drugs were subjected to screening for inhibitory activity on K^+ contracture of guinea pig taenia coli, and significant activity was observed in 17 crude drugs. Chemical investigations of two crude drugs, Kakko and Ubai, which originate from *Pogostemon cablin* and *Prunus mume*, respectively, were undertaken, and patchouli alcohol (I) and 5-(hydroxymethyl)-2-furaldehyde (II) were identified as their active principles, respectively.

Keywords Calcium antagonist; *Pogostemon cablin*; *Prunus mume*; patchouli alcohol; 5-(hydroxymethyl)-2-furaldehyde; Chinese crude drug

Introduction

It is well established that Ca^{2+} is one of the most important mediators in living organisms, and plays critical roles in a number of biological reactions, for instance, the secretion of neurotransmitters, activation of various key enzymes and most notably contraction of muscles. Drugs that act to alter Ca^{2+} levels in cells are thus expected to induce various physiological effects. The so-called Ca^{2+} antagonists are perhaps the most well-known drugs among those acting to affect cellular Ca^{2+} content, and are currently defined as a group of drugs that inhibit slow transsarcolemmal inward Ca^{2+} current without affecting the Na^+-dependent excitatory process in smooth muscle contraction.[3)] Recently it has been reported that Ca^{2+} antagonists also exhibit potent inhibitory activity on Ca^{2+} influx responsible for excitation and contraction in the electromechanical coupling, in particular, of myocardinal and vascular muscles.[4)] These findings have led to a wide clinical use of Ca^{2+} antagonists as therapeutic agents for the treatment of coronary heart diseases and hypertension.[5)] Nowadays the Ca^{2+} antagonist assay is an indispensable tool for the screening and development of new cardiovascular drugs.

We are currently working on a long-term research project to isolate biologically active principles from Chinese medicinal drugs. One of the advantages of selecting traditional medicinal drugs as subjects for chemical investigation is that they are expected to be sources of biologically active principles, having been screened through practical use in the health care of human beings for centuries. In China and its surroundings, a wide variety of plant materials, reflecting a rich flora in this area, have been extensively exploited as sources of crude drugs for folk medicine. However, in reality, not much is known about the active principles of Chinese crude drugs that would validate their use in traditional medicine in terms of modern pharmacology. One of the difficulties in dealing with Chinese medicinal drugs is that their biological activities are, in most cases, too mild and complex to detect by means of conventional *in vivo* screening tests, and this has long hampered a scientific investigation into them. This may be in part ascribed to one constituent canceling out or disturbing an effect of another, since a decoction of crude drugs, usually consisting of more than five, contains a wide variety of constituents that can act on different receptors simultaneously. One approach being undertaken by us to overcome these difficulties and to identify as many active principles as possible in crude drugs is randomly to subject crude drugs to *in vitro* bioassay irrespective of their traditional use. Under this research program we have obtained satisfactory results in the isolation of biologically active principles from crude drugs using pharmacological probes such as animal isolated organs[1,6,7)] and enzymes,[8-10)] and furthermore, in several cases, the pharmacological activities of some active principles were found to be consistent with the traditional uses of the crude drugs from which they were isolated.[6,9)]

Ca^{2+} antagonists have not found significant clinical use in diseases other than those related to cardio-vascular disorder, but could have a broad spectrum of pharmacological activity on the basis of their marked inhibition of Ca^{2+} influx. In this respect the Ca^{2+} antagonist assay would be useful as a pharmacological tool for the random screening of crude drugs to evaluate their biological activities, which are often difficult to specify in modern pharmacological terms. Interestingly, to our knowledge, there seems to be no common structural feature that characterizes the Ca^{2+} antagonists in terms of structure–activity relationships,[11)] and thus the prospects seem good for encountering new lead compounds for the development of Ca^{2+} antagonists among natural products. These circumstances coupled with recent reports on the isolation of compounds with Ca^{2+} antagonist activity from natural sources[12-14)] encouraged us to introduce the Ca^{2+} antagonist assay into our research program. The present paper describes the results of Ca^{2+} antagonist assay on hot aqueous extracts of 134 crude drugs, and identification of active principles contained in Kakko (藿香; patchouli; the aerial part of *Pogostemon cablin*) and Ubai (烏梅; smoked unripe fruits of *Prunus mume*).

Materials and Methods

General All melting points were measured on a Yanagimoto hot-stage melting point apparatus and are uncorrected. Spectral data were obtained using the following apparatus: proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra with a JEOL FX-100 spectrometer (^{1}H, 100 MHz; ^{13}C, 25 MHz) with tetramethylsilane (TMS) as an internal standard; mass spectra (MS) with a JEOL JMS-DX300 mass spectrometer; infrared (IR) spectra with a JASCO DS-701G spectrometer. Optical rotations were measured with a JASCO DIP-140 digital polarimeter. The isometric and isotonic contractions of muscles were recorded on a

rectigraph (8K-11; San-ei Sokki, Tokyo) through a Shinko U-Gage tension transducer and a strain amplifier (6M62; San-ei Sokki, Tokyo).

Materials All crude drugs used here were purchased from either Uchida Pharmacy for Sino-Japanese Medicine (Tokyo, Japan) or Kinokuniya Pharmacy for Kampo Medicine (Tokyo, Japan).

Assay Procedure for Inhibitory Activity on K^+ Contracture Taenia coli strips were dissected from ceca of guinea pigs weighing 300 to 650 g. The isolated strip was mounted in a 10 ml organ bath filled with Krebs solution (pH adjusted to 7.2) consisting of the following ingredients (concentration, mM): NaCl (120.9), KCl (5.9), $CaCl_2$ (2.5), $MgCl_2$ (1.2), $NaHCO_3$ (14.4), NaH_2PO_4 (1.2) and glucose (11.5). The bath temperature was kept at 37 °C and the bath was continuously aerated with 95% O_2 and 5% CO_2. The mechanical response of the muscles was isometrically recorded. After being equilibrated in Krebs solution for 30 min, the taenia coli was treated with hypertonic 40 mM KCl to induce K^+ contracture, and then samples were administered cumulatively. In evaluating the inhibition of K^+ contracture, the contraction of taenia coli by 40 mM KCl was taken as 0% inhibition, and the relaxation of the taenia coli induced by adding verapamil at the concentration of 10^{-5} M was taken as 100% inhibition.

Assay Procedure for Inhibitory Activity on Ca^{2+}-Induced Contraction The inhibitory activity of samples on Ca^{2+}-induced contraction of muscles was measured by the Magnus method using spiral strips of rat aortae. Male Wistar rats weighing 300 to 350 g were sacrificed, and thoracic aortae were dissected out. The spiral strip prepared from the isolated smooth muscle was mounted in a bath filled with the modified Krebs–Henseleit solution (pH adjusted to 7.4) with the following ingredients (concentration, mM): NaCl (118), KCl (4.7), $CaCl_2$ (1.8), $MgSO_4$ (1.2), NaH_2PO_4 (1.2) and glucose (11.1). The organ bath was kept at 37 °C and continuously bubbled with 95% O_2 and 5% CO_2. The mechanical response of muscles was isotonically recorded with an initial tension of 1 g loaded. After being equilibrated in the modified Krebs–Henseleit solution for 1 h, the strip was immersed in Ca^{2+}-free solution containing 3 mM ethyleneglycol-bis(β-aminoethylether)-*N*,*N*-tetraacetic acid (EGTA) for 15 min. When the strip was relaxed, it was suspended in Ca^{2+}-free solution with high K^+ concentration (123 mM KCl, 1.2 mM $MgCl_2$, 35 mM $KHCO_3$, 1.2 mM KH_2PO_4 and 11.1 mM glucose). Then Ca^{2+} was added cumulatively 5 min after administration of test samples. The pA_2 values were calculated from the dose–response curves according to Schild's method.[15]

Test Samples Hot aqueous extracts of crude drugs submitted to the primary assay were prepared as follows: ten grams of each crude drug was extracted with 100 ml of boiling water, and the filtrate was lyophilized. The dried extracts were redissolved in water, and their Ca^{2+} antagonist activity was tested with final concentrations of 3×10^{-4}, 10^{-3}, 3×10^{-3} g/ml. The use of organic solvents, which disturbed the assay significantly, was avoided. Water-insoluble samples were solubilized by the addition of polyvinylpyrrolidone (PVP, MW 40000), which had a negligible effect in the assay systems tested.

Isolation of I from Kakko The commercially available crude drug originated from *Pogostemon cablin* (500 g) was extracted successively with *n*-hexane, chloroform, methanol and water. Each extract was assayed for Ca^{2+} antagonist activity at a dose of 1.5×10^{-4} g/ml, and results were as follows: *n*-hexane extract (7.4 g, 100%); chloroform extract (4.7 g, 100%); methanol extract (18.5 g, 93%) and water extract (31.7 g, −18%). The *n*-hexane extract (7.4 g) was subjected to silica gel chromatography with a chloroform–methanol gradient. The Ca^{2+} antagonist activity of each fraction was monitored according to the assay procedure described above. Most of the activity was found in fractions eluted with chroloform–methanol (99:1). The active fractions showing 100% inhibition at 1×10^{-4} g/ml were combined (2.6 g), and rechromatographed on silica gel with *n*-hexane–ethyl acetate to give a crystalline product (I) (135 mg) as an active principle.

Isolation of II from Ubai The extraction of the commercially available crude drug originated from *Prunus mume* (500 g) proceeded as stated above, and the results of Ca^{2+} antagonist activity of each fraction were as follows (assayed at a dose of 1.5×10^{-4} g/ml): *n*-hexane extract (5.0 g, 39%); chloroform extract (4.2 g, 88%); methanol extract (173.4 g, 13%) and water extract (31.3 g, 14%). The chloroform extract (47.5 g) obtained from 3 kg of crude drug was partitioned between aqueous methanol and *n*-hexane. The methanol part (16 g) was repartitioned between water and ethyl acetate. The most potent Ca^{2+} antagonist activity was observed in the ethyl acetate part (10.4 g; 76% inhibition at 6×10^{-4} g/ml), which was repeatedly chromatographed on silica gel to afford a colorless oil (II) (100 mg) as an active principle.

Patchouli Alcohol (I) Colorless needles after sublimation, mp 54—56 °C. $[\alpha]_D^{20}$ −124° ($CHCl_3$, $c=0.22$). IR ν_{max}^{KBr} cm^{-1}: 3620 (OH), 2960 (CH_3), 2940 (CH_2). ^{1}H-NMR (100 MHz, $CDCl_3$) δ: 0.82 (3H, d, $J=6$ Hz, CH_3), 0.88 (3H, s, CH_3), 1.09 (6H, s, $2\times CH_3$), 1.0—2.0 (14H, m). ^{13}C-NMR (25 MHz, $CDCl_3$) δ: 18.5 (CH_3), 20.6 (CH_3), 24.3 (CH_3 and CH_2), 24.5 (CH_2), 26.8 (CH_3), 28.0 (CH), 28.5 (CH_2), 28.8 (CH_2), 32.6 (CH_2), 37.6 (–C–), 39.0, (CH), 40.0 (–C–), 43.6 (CH), 75.4 (–C–OH). EI-MS *m/z* (rel. int., %): 222 (M^+, 100), 161 (53), 138 (83), 125 (58), 109 (51).

5-(Hydroxymethyl)-2-furaldehyde (II) Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 3000, 2830, 1675, 1518, 1186. ^{1}H-NMR (100 MHz, $CDCl_3$) δ: 2.54 (1H, s, OH), 4.71 (2H, s, CH_2O), 6.51 (1H, d, $J=3.4$ Hz), 7.22 (1H, d, $J=3.4$ Hz), 9.58 (1H, s, CHO). HR-MS Calcd for $C_6H_6O_3$: 126.0316. Found: 126.0316.

Results and Discussion

Hot aqueous extracts of Chinese medicinal drugs were tested for Ca^{2+} antagonist activity using the guinea pig taenia coli as a pharmacological probe. The primary assay was carried out with the administration of each sample at three doses. Of 134 extracts tested, potent inhibitory activity on K^+ contracture was observed in 17 crude drugs as

TABLE I. List of Chinese Medicinal Drugs with Potent Ca^{2+} Antagonist Activity

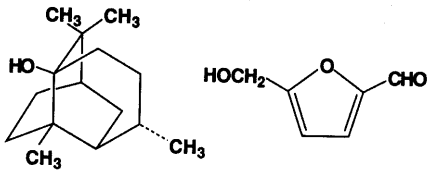
Name of crude drug (Japanese name)	Origin of crude drug	Part used	Ca^{2+}-Antagonist activity (%) Doses ($\times10^{-4}$ g/ml) 3	10	30
Inchinkou (茵蔯蒿)	*Artemisia capillaris*	Flower	47	89	93
Ubai (烏梅)	*Prunus mume*	Fruit	38	66	94
Ougon (黄芩)	*Scutellaria baicalensis*	Root	83	—	—
Oubaku (黄柏)	*Phellodendron amurense*	Bark	67	91	—
Ouhi (桜皮)	*Prunus jamasakura*	Bark	84	—	—
Kakko (藿香)	*Pogostemon cablin*	Whole plant or aerial part	17	75	91
Kikka (菊花)	*Chrysanthemum morifolium*	Flower	12	57	92
Kinsensou (金銭草)	*Desmodium styracifolium*	Whole plant	29	65	88
Kouboku (厚朴)	*Magnolia obovata*	Bark	15	84	—
Goboushi (牛蒡子)	*Arctium lappa*	Seed	25	100	—
Jashoushi (蛇床子)	*Cnidium monnieri*	Fruit	17	53	84
Seihi (青皮)	*Citrus unshiu*	Pericarp	64	95	—
Zenko (前胡)	*Peucedanum praeruptorum*	Root	85	93	—
Daiou (大黄)	*Rheum palmatum*	Rhizome	25	69	97
Takusha (沢瀉)	*Alisma orientale*	Rhizome	25	51	84
Nikuzuku (肉豆蔻)	*Myristica fragrans*	Seed	17	79	98
Hokotsushi (補骨脂)	*Psoralea corylifolia*	Seed	23	87	—

—, not tested.

summarized in Table I. Few of these crude drugs have been used in traditional medicine as a remedy for hypertension or cardio-vascular diseases, for which Ca^{2+} antagonists have proved to be most effective. Similar remarks were made independently by another group.[13] This not only illustrates one of the complexities with Chinese crude drugs, that their traditional use cannot necessarily be construed in terms of their biological activities detected in a single assay system, but also clearly illustrates the significance of our approach of randomly assaying biological activities of crude drugs, irrespective of their traditional use, by use of various pharmacological probes.

The active principles in Goboushi (牛蒡子; fruits of *Arctium lappa*) were identified as lignans, and their structure–activity relationships were discussed in our previous paper.[1] Recently Ca^{2+} antagonistic principles in Zenko (前胡; roots of *Peucedanum praeruptorum*),[12] Jashoushi (蛇床子; seeds of *Cnidium monnieri*)[13] and Kouboku (厚朴; bark of *Magnolia obovata*)[14] have been identified. On the other hand, the inhibitory activity by Seihi (青皮; Citrus peels) can be ascribed to a sympathomimetic substance, (−)-synephrine, which directly acts as a relaxant on smooth muscles and has been found abundantly in this crude drug.[6] Taking into account recent advances in chemical studies on crude drugs listed in Table I, we finally selected two crude drugs, Ubai and Kakko, to undertake chemical studies guided by assay of Ca^{2+} antagonist activity.

The commercially available crude drugs were successively extracted with solvents in the order of increasing polarity according to the method described before.[7] The assay of each extract for Ca^{2+} antagonist activity revealed that the activity occurred in the less polar part of the extracts of both crude drugs. Hence the *n*-hexane extract of Kakko, and the chloroform extract of Ubai were selected for further detailed chemical studies.



I

II

The *n*-hexane extract (7.4 g) of Kakko was subjected to repeated chromatographic separation on silica gel followed by sublimation to afford compound I with potent Ca^{2+} antagonist activity. Compound I was obtained as optically active colorless needles, $[\alpha]_D$ −124°, mp 54—56 °C. The ^{1}H-NMR spectrum indicated the presence of four methyls [δ 0.82 (3H, d, J=6 Hz), 0.88 (3H, s) and 1.09 (6H, s)], and the ^{13}C-NMR spectrum confirmed the presence of five methylenes, three methines and three quaternary carbons. The IR spectrum showed an absorption band of OH at 3620 cm^{-1}. From these findings compound I was readily elucidated as a sesquiterpene, patchouli alcohol (I), which is one of the main constituents in patchouli,[16] and finally identified by mixed melting point determination and IR spectral comparison with an authentic sample. The pA_2 and IC_{50} of patchouli alcohol (I) were calculated to be 5.95 and 4.7×10^{-5} M, respectively, from the dose–response

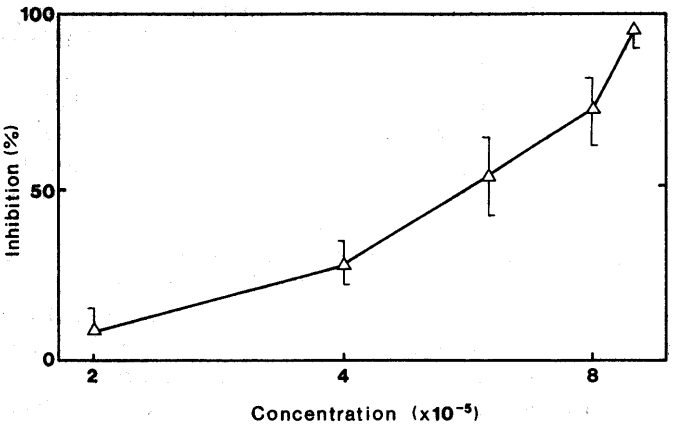


Fig. 1. Inhibitory Activity of Patchouli Alcohol on K^+-Induced Contraction of Guinea Pig Taenia Coli

Points are means of 4 experiments. Vertical bars show S.E.

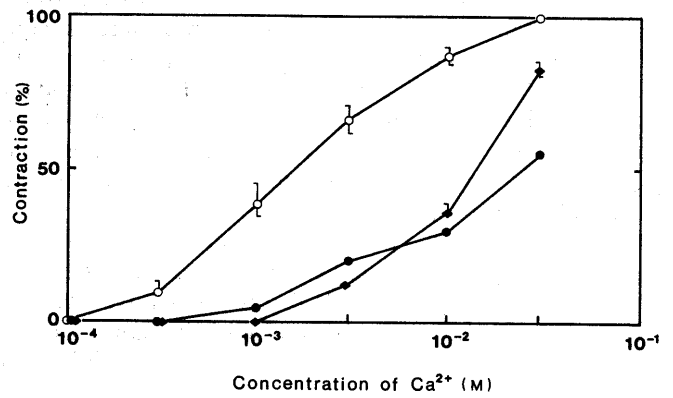


Fig. 2. Inhibitory Activity of Patchouli Alcohol on Ca^{2+}-Induced Contraction of Rat Aortae

Points are means of 4 experiments except for the control (means of 10 experiments). Vertical bars show S.E. ○, control; ●, patchouli alcohol 2×10^{-5} M; ◆, verapamil 1×10^{-7} M.

curves shown in Figs. 1 and 2. Its relative potency of Ca^{2+} antagonist activity was estimated at approximately 1/150 of that of verapamil, which is a notable Ca^{2+} antagonist of great clinical value and whose pA_2 value was found to be 7.75 in the identical assay system. Kakko is one of the traditional Chinese folk medicines used mainly for the treatment of dyspepsia, vomiting, diarrhea and poor appetite. Excessive contraction of digestive organ muscles may be a partial cause of vomiting and diarrhea, and thus the Ca^{2+} antagonist is expected to alleviate those symptons by depressing excessive excitation of smooth muscles as a result of inhibition of inward Ca^{2+} influx through the cell membranes. In this context, patchouli alcohol (I), which occurs abundantly in Kakko (>0.1%), may have pharmacological significance as one of the active principles, in view of its use in traditional medicine.

The chloroform extract of Ubai afforded compound II as an active principle after partition followed by repeated chromatographic separation. Its molecular formula was determined as $C_6H_6O_3$ by high-resolution mass spectrometry (HR-MS). The ^{1}H-NMR spectrum indicated the presence of carbinol protons [δ 4.71 (2H, br s)], a pair of AB doublets [δ 6.51 (1H, d, J=3.4 Hz) and 7.22 (1H, d, J=3.4 Hz)] and an aldehyde proton [δ 9.58 (1H, s)], and led

readily to the assignment of its structure as 5-(hydroxymethyl)-2-furaldehyde (II). The IC_{50} of compound II was calculated to be 5.8×10^{-3} M from the dose–response curve. Although II was less active than patchouli alcohol (I) by two orders of magnitude, it showed reproducible inhibitory activity on K^+ contracture. Ubai is traditionally used for the treatment of coughing, vomiting and diarrhea, for which Kakko is also often prescribed, and thus the presence of a Ca^{2+} antagonist in this crude drug may have significance, as in the case of Kakko. There has been a report on the isolation of 5-(hydroxymethyl)-2-furaldehyde (II) from natural products,[17] but as far as Ubai is concerned, it is considered to be an artefact arising from transformation of sugars, rather than a natural product, since sucrose gives rise to compound II on treatment with alkali followed by acid.[18] Interestingly, the commercially available form of Ubai is prepared by special processing in which unripe fruits of *Prunus mume* are immersed in ash–water mixture, and then smoked according to the traditional procedure.[19] Since the above processing is considered to be chemically equivalent to "treatment with alkali," it is presumed that compound II is a secondary product derived from the successive treatment of sugars, which occur abundantly in the fruits, with "ash–water" and organic acids, which also abound in fruits, during the processing. The processing of crude drugs prior to use is called "Shuji (修治)" in traditional term, and has been practiced purportedly for either quenching the toxicity or upgrading the quality of crude drugs. The above example may provide a scientific basis for one of the many roles of "Shuji," which have been understood only on an empirical basis.

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References and Notes

1) A part of this study was presented at the 104th Annual Meeting of the Pharmaceutical Society of Japan held in Sendai, March 28th through 30th, 1984 (see p. 130, 131 in the Abstracts of Papers), and also at the UNESCO Regional Workshop at Seoul in Korea, September 20th through 25th, 1987 (see p. 43 in *Proceedings of Unesco Regional Workshop on in Vitro Bioassay*). This is the fourth report in a series of "Chemical Studies on Traditional Medicines Acting on Animal Isolated Organs." Previous report: K. Ichikawa, T. Kinoshita, S. Nishibe and U. Sankawa, *Chem. Pharm. Bull.*, **34**, 3514 (1986).
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