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Inhibitory effects of Senkyu-chacho-san and Cnidii Rhizoma on catechol-*O*-methyltransferase

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In the treatment for the symptoms of Parkinson's disease, the catechol-*O*-methyl transferase (COMT) inhibitors are expected to mitigate side effects, such as wearing-off, which are caused by long-term administration of L-DOPA. It has been reported that Senkyu-chacho-san causes dopamine increase in a rat striatum. However, the detailed mechanism has not been clarified about the mechanism. Search for components inhibiting COMT led to the discovery of active compounds contained in Cnidii Rhizoma (Senkyu). The hot water extract from the Cnidii Rhizoma was subjected to distribution or gel filtration chromatography to give ferulic acid (1), sinapic acid (2), 5-hydroxy-ferulic acid (3), and chlorogenic acid (4). Their structures were determined by spectroscopic analyses, particularly by extensive 1D and 2D NMR studies. Isolation of 3 is the first report from Cnidii Rhizoma. These compounds can be regarded as substrate-mimetics, and the number of the hydroxyl group in benzene nucleus seems to have a significant effect on inhibitory activity against COMT. Compound 3 inhibited COMT in a competitive manner and its *Ki* value was calculated 43 μM from Lineweaber-Burk plots. These results suggest that the phenylpropanoides, which are the components of Cnidii Rhizoma, might raise the dopamine level in the brain.

Key words Parkinson's disease, Catechol-*O*-methyltransferase, Senkyu-chacho-san, 5-Hydroxy-ferulic acid.

Abbreviations COMT, catechol-*O*-methyl transferase; L-DOPA, L-3,4-dihydroxyphenylalanine; COSY, correlated spectroscopy; HMBC, heteronuclear multiple bond correlation.

Introduction

Parkinson's disease is a progressive neurological disorder caused by a loss of nerve cells in the substantia nigra and a small area deep within the brainstem. It is thought that the main disorders of Parkinson's disease, such as the trembling, rigidity, slowness of operation, and a posture maintenance obstacle, are based on the lack of dopamine in a nigrostriatal system, and the oral medication of the precursor L-3,4-dihydroxyphenylalanine (L-DOPA) is carried out instead of dopamine which does not pass through a blood brain gateway.[1,2] However, the side effects, such as a wearing-off phenomenon, an on-off phenomenon, involuntary movement, and moral condition, mostly appear by a long-term administration of large quantities of L-DOPA. A wearing-off phenomenon is seen in about 30% of patients in three years after L-DOPA internal administration, and it has become a serious problem in a L-DOPA supplement treatment.[3,4] L-DOPA is largely converted into 3-*O*-methyldopamine by catechol-*O*-methyl transferase (COMT).[5] Thus, selective COMT inhibition improves the bioavailability of levodopa and allows a decrease in its dose as shown in recent clinical trails.[6] Recently, new catechol derivatives that show potent and selective COMT inhibition both *in vitro* and *in vivo* have been synthesized. Tolcapone (Tasmar™)[7] and Entacapone (Comtan™)[8] are reversible inhibitors with 5-nitrocatechol structures. In recent years, there are some reports that Kampo medicines are effective for the improvement of the mental aberration, trembling and rigidity, which are observed during the L-DOPA treatment of Parkinson's disease.

Hange-koboku-to, Rikkunshi-to, Shigyaku-san, Yoku-kan-san, Shakuyaku-kanzo-to, and Senkyu-chacho-san are supposed to be effective for Parkinson's disease.[9-14] Among these, Hange-koboku-to, Yoku-kan-san and Shakuyaku-kanzo-to are considered to be the prescription focused on the improvement of a trembling or rigidity and such effect of their components have also been studied. Pinelliae Tuber (Hange) has the nerve system-suppressing effect and the sedative and anticonvulsant effects. Magnocurarine, magnolol, and honokiol, which are components of Magnoliae Cortex (Koboku), show muscle relaxant, antispasmodic effect, and sedative effect as well.[15,16] On the other hand, Paeoniae Radix (Shakuyaku) containing paeoniflorin shows the painkilling, anticonvulsant, peripheral blood vessel extension, and anti-inflammation actions.[17] Glycyrrhizae Radix (Kanzo) is often used together with Paeoniae Radix since the effects described above are enforced by co-administration.[18,19] However, these studies are limited to the use of Kampo medicine as an expectant treatment. There are some interesting reports on the use of Rikkunshi-to for Parkinson's disease. Kawakami *et al.*[20] has examined the effect of Rikkunshi-to on the metabolism of dopamine and serotonin (5-HT). They have measured the concentration in the spine of 3-methoxy-4-hydroxyphenylethylene glycol (HMPG), norepinephrine, and 5-HT, which are the main metabolites of 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and dopamine, respectively, using HPLC. Consequently, there was no significant change in the

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concentration of HVA and HMPG. On the other hand, the 5-HIAA concentration significantly increased. The 5-HT metabolism associates with the development of L-DOPA-induced dyskinesia in Parkinson's disease patients. Muramatsu *et al.*[13] and Shizuma *et al.*[14] have reported that Senkyu-chacho-san increased dopamine in the striatum and showed a significant improvement in motor performance with respect to the motor scores of the Unified Parkinson's Disease Rating Scale (UPDRS).

In this paper, we describe the inhibitory effects of six Kampo medicines used for Parkinson's disease and four phenylpropanoids isolated from one of the constituting crude drugs, Cnidii Rhizoma, on COMT, and the structural determination of the active components.

Materials and Methods

Preparation of Kampo herbal medicines and their extracts for assay. The recipes of formulation in this study are as follow : Hange-koboku-to ; Pinelliae Tuber (8.0 g), Hoelen (Bukuryo ; 5.0g), Magnoliae Cortex (3.0g), Perilliae Herba (Soyo ; 2.0 g), Zingiberis Rhizoma (Shokyo; 1.0 g) : Senkyu-chacho-san ; Menthae Herba (Hakka ; 4.0g), Cyperi Rhizoma (Kobushi ; 4.0 g), Chidii Rhizoma (3.0g), Schizonepetae Herba (Keigai ; 3.0 g), Saposhnikoviae Radix (Bofu ; 2.0 g), Thea Foliym (Chayo; 2.0 g), Notopterygii Rhizoma (Kyokatsu ; 1.0 g), Angelicae Dahuricae Radix (Byakushi ; 1.0 g), Glycyrrhizae Radix (1.0 g) : Shakuyaku-kanzo-to ; Paeoniae Radix (3.0 g), Glycyrrhizae Radix (1.0 g) : Shigyaku-san ; Bupleuri Radix (Saiko ; 5.0 g), Paeoniae Radix (5.0 g), Aurantii Fructus Immaturus (Kijitsu ; 4.0g), Glycyrrhizae Radix (3.0 g) : Rikkunshi-to ; Ginseng Radix (Ninjin, 4.0g), Pinelliae Tuber (8.0 g), Atractylodis Rhizoma (Byakujutsu ; 4.0g) ; Hoelen (5.0g), Zizyphi Fructus (Taiso; 2.0g), Aurantii Nobilis Pericarpium (Chinpi ; 2.0g), Glycyrrhizae Radix (1.0 g), Zingiberis Rhizoma (1.0 g) : Yokukan-san ; Atractylodis Rhizoma (4.0g), Hoelen (4.0g), Angelicae Radix (Toki ; 4.0g), Uncaria Ramulus Et Uncus (Chotoko ; 4.0g), Chidii Rhizoma (3.0g), Glycyrrhizae Radix (1.5 g). These crude drugs were purchased from Tochimoto Tenkaido Co Ltd. (Osaka, Japan) and Uchida Wakanyaku Co Ltd. (Tokyo, Japan). Each Kampo medicine or Kampo herbal medicine (20g) was extracted with distilled water (600ml) for 40 minutes and centrifuged (1,000×g, 20min). The supernatant was lyophilized to give a powder.

General methods. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Jeol JNM-GX 400 spectrometer. Chemical shifts are expressed in ppm downfield from tetramethylsilane (TMS) in CD_3OD as an internal standard. The proton and carbon signals was assigned from extensive homonuclear decoupling experiments, DEPT, ^{1}H-^{13}C COSY, and HMBC spectral data. FABMS were measured on a Jeol JMS-SX 102A spectrometer.

Preparation of *O*-methyl transferase from rat liver. Catechol-*O*-methyltransferase (COMT) was prepared from rat liver according to the method of Axelrod *et al.*[21] Wistar male rats (8 weeks) were stunned and exsanguinated, and the liver was rapidly excised, washed with chilled 0.25M sucrose (pH 7.0) solution, and minced with scissors. The minced livers were homogenized with 4 volumes of isotonic KCl and centrifuged at 4,200×g for 20 minutes. The soluble supernatant fraction was centrifuged at 78,000×g for 30 minutes. The resulting supernatant was adjusted to pH 5.0 with 1 M acetic acid, allowed to stand for 10 minutes, and centrifuged at 4,700×g for 10 minutes. To 300 ml of the supernatant fraction was added 52 g of ammonium sulfate (30 % saturation). The precipitate was discarded and 34 g of ammonium sulfate (50 % saturation) were further added to the supernatant fraction. After being centrifuged at 4,700 ×g for 10 minutes, the precipitate was used as the enzyme source of COMT. All purification procedures were carried out at 0-4℃.

Enzyme assay. Catechol-*O*-methyl transferase activity was determined by measuring the amount of metanephrine formed from epinephrine. The reaction mixture, containing 10 μl of the enzyme solution, 34 μl of inhibitor solution or water, 6 μl of 500 mM magnesium sulfate, 30 μl of 5 mM α-adenocyl methionine, 70 μl of 6 mM l-epinephrine and 150 μl of phosphate buffer (pH 7.8), was incubated for 10 min at 37℃. The reaction was stopped by adding 150 μl of 0.5 M borate buffer (pH 13). Metanephrine was extracted with 9 ml of ethylene dichloride containing 2 % isoamyl alcohol, returned to 1.5 ml, 0.1 M HCl, and measured fluorometrically at 335 nm after activation at 285 nm.

Kinetics of inhibition. Enzyme inhibition modes and *K*i values for the 5-hydroxy-ferulic acid was determined from Lineweaver-Burk plots.

Extraction and isolation. The Cnidii Rhizoma (400g) from a commercial source were extracted with hot water for 40 min. The hot water extract was filtered through Celite and the filtrate was extracted three times with ethyl acetate. After concentration of the ethyl acetate layer, the residue was suspended in H_2O, extracted three times with MeOH, and divided into MeOH soluble and insoluble fractions. The MeOH soluble fraction was chromatographed over a Sephadex LH-20 column (200 ml, Pharmacia Fine Chemical) with MeOH as eluant. The MeOH elute was divided into two pools A (271.4 mg) and B (176.1 mg). Pool A was chromatographed on a Dowex 1-X2 column (250 ml, OH^- form) with H_2O as eluant to give chlorogenic acid (**4**, 11.7 mg). Pool B was purified by preparative TLC on Silica Gel-60F_{254} (E. Merck) developed with MeOH- H_2O (7 : 3) and finally purified by Sephadex LH-20 (250 ml) with MeOH as eluant to give ferulic acid (**1**, 97.2 mg), sinapic acid (**2**, 17.3 mg), and 5-hydroxy-ferulic acid (**3**, 34.1 mg). 5-Hydroxy-ferulic acid (4,5-dihydroxy-3-methoxycinnamic acid) (**3**). HRFAB-MS *m/z* 211.0611 $[M+H]^+$ ($C_{10}H_{11}O_5$ requires 211.1943); ^{1}H-NMR (400 MHz, CD_3OD) δ/ppm = 3.86 (3H, s, H-10), 6.25 (1H, d, *J* = 15.8 Hz, H-8), 6.72 (1H, d, *J* = 1.8 Hz, H-6), 6.74 (1H, d, *J* = 1.8 Hz, H-2), 7.51 (1H, d, *J* = 15.8 Hz, H7); ^{13}C-NMR (100 MHz, CD_3OD) δ/ppm = 56.7 (C-10), 104.9 (C-2), 110.2 (C-6), 116.0 (C-8), 126.7 (C-1), 138.3 (C-4), 146.8 (C-5), 147.3 (C-7), 149.8 (C-3) and 170.9 (C-9).

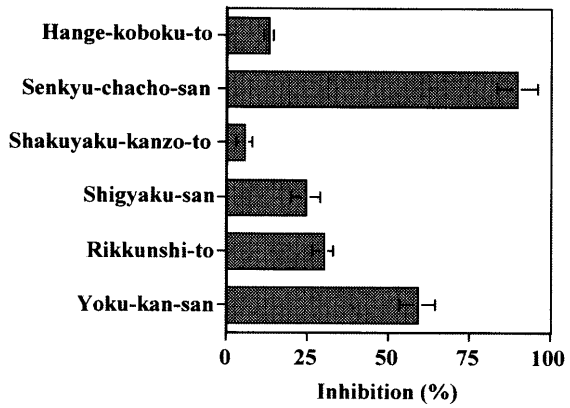

Fig. 1 Effect of Kampo medicines on catechol-*O*-methyltransferase activity
Kampo medicines (200 μg/ml) were preincubated with catechol-*O*-methyltransferase for several minutes and then the reaction was initiated by addition of epinephrine as substrate. After an incubation of 20 min, the reaction was stopped by addition of 150 μl of 0.5 M borate buffer (pH 13), and the formation of metanephrine was fluorometrically measured at 335 nm after activation at 285 nm. The data are expressed relative to control incubations and as mean±S.E.M. (n=3).

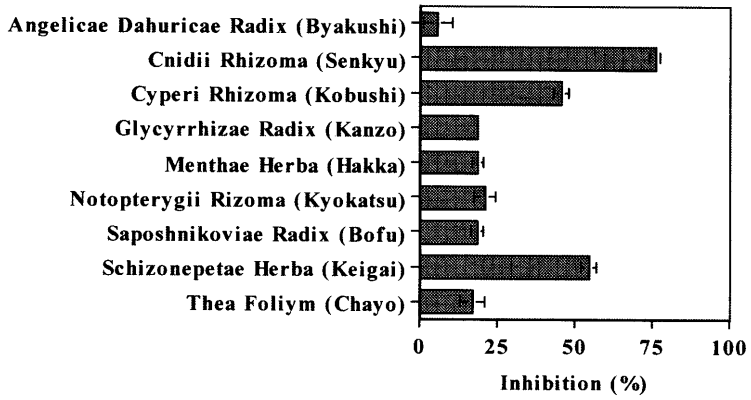

Fig. 2 Effect of the constituting herbal medicines of Senkyu-chacho-san on catechol-*O*-methyltransferase activity
Herbal medicines (20 μg/ml) were preincubated with catechol-*O*-methyltransferase for several minutes and then the reaction was initiated by addition of epinephrine as substrate. After an incubation of 20 min, the reaction was stopped by addition of 150 μl of 0.5 M borate buffer (pH 13), and the formation of metanephrine was measured fluorometrically at 335 nm after activation at 285 nm. The data are expressed relative to control incubations and as mean±S.E.M. (n=3).

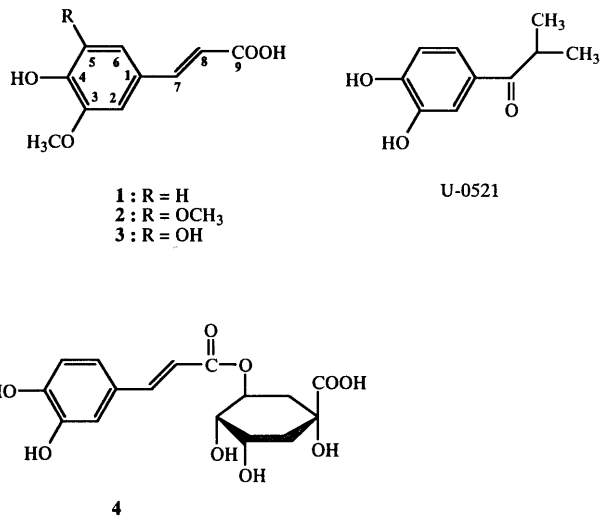

Fig. 3 Structures of phenylpropanoids isolated from *Cnidium officinale*.

Results

Effect of Kampo medicines and the components on catechol-*O*-methyltransferase. We first examined the effects of six Kampo medicines, which were used for Parkinson's disease, on catechol-*O*-methyltransferase (COMT) activity. In order to remove the non-specific inhibition factor, 1 mg/ml BSA (final dose) was added to the reaction system. As shown in Fig.1, Senkyu-chacho-san and Yoku-kan-san at a concentration of 200 μg/ml inhibited COMT activity by 89.8% and 59.2%, respectively. Senkyu-chacho-san inhibited COMT in a dose-dependent manner (100 μg/ml, 48.8% ; 50 μg/ml, 23.5%). Interestingly, Shigyaku-san, Yoku-kan-san and Shakuyaku-kanzo-to, which mainly consist of Paeoniae Radix and Glycyrrhizae Radix, showed no significant inhibition (data not shown).We further examined the COMT inhibitory activity of the constituting crude drugs of Senkyu-chacho-san, and found that Cnidii Rhizoma showed 75.9% inhibition at the concentration of 20 μg/ml, whereas Cyperi Rhizoma (Kobushi) and Schizonepetae Herba (Keigai) showed much weaker inhibition than did Cnidii Rhizoma (Fig.2). These results suggest that components responsible for COMT inhibition by Senkyu-chacho-san were mainly contained in Cnidii Rhizoma.

Isolation of active components and their structure determinations. A hot water extract of the dry rhizomes (400 g) of Cnidii Rhizoma was chromatographed with Sephadex LH-20 and various ion-exchange resins to give compounds **1** (97.2 mg), **2** (17.3 mg), **3** (34.1 mg), and **4** (11.7 mg). The structures of compounds isolated from Cnidii Rhizoma were determined from combined ^{1}H and ^{13}C NMR spectroscopic data, including extensive homonuclear decoupling experiments, NOE enhancements, and two-dimensional HMQC experiments. The optical rotation, FAB-MS and NMR spectral data of compounds **1, 2** and **4** were in accord with those of ferulic acid, sinapic acid and chlorogenic acid, respectively (Fig. 3). The ^{13}C NMR (100 MHz) spectra in CD_3OD of **3** gave nine resonances. This data was very similar to those of sinapic acid. High-resolution FAB-mass spectrometry (FAB-MS) (glycerol matrix) of **3** gave an $[M+H]^+$ ion at *m/z* 211.0611. The spectral data described above imply that compound **3** is methoxycinnamic acid. The ^{1}H NMR data, together with information from extensive decoupling experiments and two-dimensional ^{1}H-^{13}C COSY spectral data, defined the complete connectivity of the carbon and hydrogen atoms. The location of the methoxy group was determined to be C3 since the coupling patterns of H2 and H6 in the phenyl group were not equal and a long-range coupling was observed between H6 and C7 β-carbon. Therefore, the structure of **3** was determined to be 5-hydroxy-ferulic acid (trans-4,5-dihydroxy-3-methoxycinnamic acid).

Table 1 Concentration of compounds **1-4** (μM) giving 50% inhibition of catechol-*O*-methyltransferase activities

Compounds	IC_{50} (μM)
Ferulic acid, **1**	1690
Sinapic acid, **2**	1290
5-Hydroxy-ferulic acid, **3**	22
Chlorogenic acid, **4**	32
U-0521	68

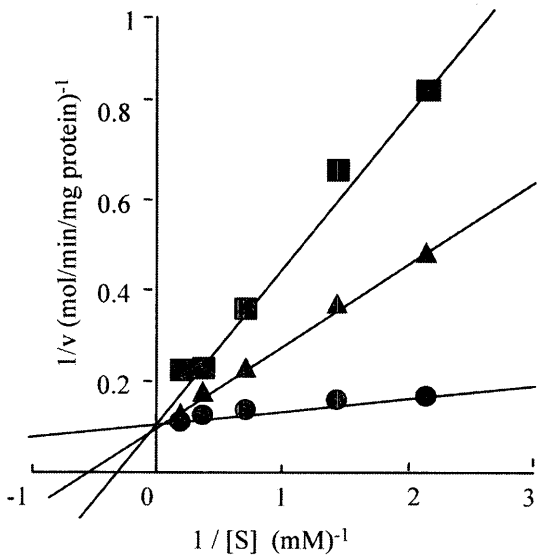

Fig. 4 Lineweaver-Burk plots of 5-hydroxy-ferulic acid inhibition of catechol-*O*-methyltransferase. The increasing concentrations of epinephrine were used to determine the *K*m and *K*i values. Concentrations of 5-hydroxy-ferulic acid were 0 (●), 332 μM (▲), and 996 μM (■). The data were plotted as 1/v against 1/S.

Catechol-*O*-methyl transferase inhibitory activities of phenylpropanoids. The IC_{50} values of four phenylpropanoids against COMT are shown in Table 1. U-0521 (3, 4-dihydroxy-2-methylpropiophenone), which is well known as a COMT inhibitor,[22] was used as standard for comparison. 5-Hydroxy-ferulic acid (**3**) showed a most potent inhibitory activity among the isolated compounds and the potency was comparable to that of U-0521. Ferulic acid (**1**) weakly inhibited COMT (IC_{50} = 1690 μM). Interestingly, 5-hydroxy-ferulic acid (**3**) has been found to be a good inhibitor of COMT (IC_{50} = 22 μM), whereas the 5-methoxy derivative, sinapic acid (**2**), was a much weaker inhibitor than 5-hydroxy-ferulic acid (**3**). These results suggest that the addition of a hydroxyl group at C5 further enhances the inhibitory potential toward COMT. Chlorogenic acid (**4**) consisting of quinic acid and caffeic acid showed good inhibition (IC_{50} = 32 μM) comparable to 5-hydroxy-ferulic acid (**3**) or U-0521. The mode of inhibition of 5-hydroxy-ferulic acid (**3**) for COMT is shown in Fig. 4. When epinephrine was used as substrate, 5-hydroxy-ferulic acid (**3**) inhibited COMT in a competitive manner, with a *K*i value of 43.2 μM.

Discussion

Although L-DOPA is used as a therapeutic agent for Parkinson's disease, it has serious side effects such as a wearing-off phenomenon, an on-off phenomenon, involuntary movement, and moral condition. In this study, we investigated inhibitory effects of six Kampo medicines used for Parkinson's disease on the catechol-*O*-methyltransferase (COMT) activity because COMT inhibition is expected to mitigate the side effects of L-DOPA. Among six Kampo medicines, Senkyu-chacho-san showed the most potent inhibitory activity against COMT. Senkyu-chacho-san has been reported to increase the dopamine level by COMT inhibition.[13,14] So, we measured the inhibitory activity of eight crude drugs constituting Senkyu-chacho-san against COMT. Consequently, Cnidii Rhizoma showed a significant inhibitory activity against COMT (Fig. 2). Search for active components in Cnidii Rhizoma led to the isolation of ferulic acid (**1**), sinapic acid (**2**), 5-hydroxyferulic acid (**3**), and chlorogenic acid (**4**). 5-Hydroxyferulic acid (**3**) and chlorogenic acid (**4**) exhibited good inhibitions against COMT, showing the IC_{50} values of 22 and 32 μM, respectively, whereas ferulic acid (**1**) and sinapic acid (**2**) were much a weaker inhibitor than compounds **3** and **4** (Table 1). The inhibitory effect of **3** on monoamine oxidase (MAO) and aromatic amino acid decarboxylase (AADC) were examined, but it exhibited no significant inhibitory activity against both enzymes (data not shown). The enzyme COMT transfers a methyl group to the OH group in the meta position of the catecolamine. Hence, it can be speculated that ferulic acid (**1**) and sinapic acid (**2**) lacking such an OH group are weak inhibitors of COMT, and 5-hydroxyferulic acid (**3**) and chlorogenic acid (**4**) regarded as the substrate analogues show the competitive inhibition.

In this study, we elucidated that the phenylpropanoides in the constituting herbal medicine of Senkkyu-chacho-san, Cnidii Rhizoma, show inhibitory activity against rat liver COMT. The phenylpropanoides have been reported to have anti-inflammatory effect, painkilling effect, and suppression of platelet aggregation.[23-25] Although these phenylpropanoides are also contained in Cimicifugae Rhizoma (Shoma), Coptidis Rhizoma (Oren), Angelicae Radix (Toki), and Asa Foetida (Agi), the phenylpropanoide content in Cnidii Rhizoma is higher than those of any herbal medicines described above. There is no report on physiological activities of 5-hydroxyferulic acid (**3**). Although it has been reported that Senkyu-chacho-san increases dopamine in the rat striatum,[13] the mechanism has not been clarified. Since the phenylpropanoides obtained in this study show the obvious inhibition against COMT, these compounds may be responsible for the rise of dopamine.

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Japanese abstract

パーキンソン病治療に汎用されている漢方薬のカテコール-*O*-メチル基転移酵素（COMT）に対する阻害効果を検討した。測定した6種の漢方方剤のうち，川芎茶調散と抑肝散に阻害活性が認められ，200 μg/ml 添加により，それぞれ89.8％，59.2％の阻害活性を示した。川芎茶調散の構成生薬に着目し同様の測定を行ったところ，川芎に強い阻害活性が認められ，20 μg/ml 添加により 75.9％の強い阻害活性が見られた。また香附子と荊芥にも中程度の阻害が認められた。パーキンソン病に用いられる方剤の多くは，四逆散や芍薬甘草湯に代表されるように芍薬と甘草をベースとするものが多いが，芍薬と甘草には顕著な COMT 阻害活性は認められなかった。川芎から活性成分の単離を試みたところ，阻害物質として ferulic acid, sinapic acid, 5-hydroxyferulic acid および chlorogenic acid が単離できた。このうちメタ位に水酸基が配位している 5-hydroxyferulic acid と chlorogenic acid には，比較的強い COMT 阻害活性が認められた。また，5-hydroxyferulic acid は Lineweaver-Burk Plot を作成して検討した結果，拮抗型の阻害様式を示した（Ki=43.2 μM)。以上の結果から，川芎茶調散はパーキンソン病治療にあたり脳内 L-DOPA あるいは dopamine レベルを増加させる可能性が示唆された。

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