芍薬甘草湯及び他の漢方処方の構成生薬がEmericella nidulansのsterigmatocystin産生に与える効果

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Effect of Shakuyaku-kanzo-to and other crude drug components of Kampo medicines on sterigmatocystin production by *Emericella nidulans*

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Keywords
crude drug; *Emericella nidulans*; Kampo medicine; Peony root; Shakuyaku-kanzo-to; sterigmatocystin

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Abstract

We investigated the production of fungal metabolites as a biological response to Kampo medicines by incubating the filamentous fungus *Emericella nidulans* IFM 60678 with Kampo medicines and analyzing the culture extracts by HPLC. *E. nidulans* IFM 60678 was found to produce sterigmatocystin (ST) upon incubation with Shakuyaku-kanzo-to. Further investigation revealed that the production of ST is stimulated by Peony root (*Paeonia lactiflora*), a component of Shakuyaku-kanzo-to. Peony root extract induced ST production in 21 of 27 (78%) non-ST-producing *E. nidulans* strains but did not induce the production of ST or aflatoxins in 22 non-ST-producing *Aspergillus flavus* strains. This result suggested that Peony root extract may induce ST production by *E. nidulans* specifically. Several other crude drugs, and especially Alisma tuber (*Alisma orientale*), Bupleurum root (*Bupleurum falcatum*), Japanese Angelica root (*Angelica acutiloba*), and Pinellia tuber (*Pinellia ternata*), potently induced ST production. Our unique and simple method might help identify new natural products and new regulatory strategies applicable to mycotoxin production.

1. Introduction

Fungi produce various secondary metabolites[1),2)] and of the metabolites isolated, some have proven useful and others are harmful mycotoxins[3),4)]. These fungal metabolites are produced by fungi as biological responses to medium components, culture temperature[5)], cultural medium pH[6)], and the addition of non-nutritional compounds such as epigenetic chemicals[7)]. These findings led us to study fungal metabolite production as a biological response of fungi to Kampo medicines, including many phytochemicals. We previously reported that the production of prenylated xanthone derivatives by *Aspergillus nidulans* CBS 112.46 (= IFM 60678) was greatly increased in the presence of Shimbu-to[8)].

Sterigmatocystin (ST) is a mycotoxin produced by fungi such as *Emericella nidulans, Aspergillus flavus,* and *Aspergillus versicolor* that causes mutagenicity in human lung and liver. In this study, we investigated the effect of 27 Kampo medicines on ST production by *E. nidulans* IFM 60678. We found that ST was produced upon incubation with 13 of the Kampo medicines, with Shakuyaku-kanzo-to (SKT) having the greatest effect. Further investigation revealed that ST production is induced by Peony root (*Paeonia lactiflora*), a component of SKT.

Herein we report the stimulation of ST production by Peony root in non-ST-producing *E. nidulans* strains and non-ST-producing *A. flavus* strains. In addition, we report the ST-inducing activity in *E. nidulans* of several crude drugs in Kampo medicines.

2. Materials and Methods

2.1. General experimental procedures

Analytical HPLC was performed using a Shimadzu Co. (Kyoto, Japan) Prominence system (pump: LC-20AD, auto-sampler: SIL-20A, detector: SPD-M20A, column oven: CTO-20A, system controller: CBM-20A, degasser: DGU-20A) with a Mightysil RP-18 column GP II (3.0 × 250 mm 5 μm, Kanto Chemical Co., Ltd., Tokyo, Japan). All fungi were fermented in an incubator (SANYO MIR-153; SANYO Electric Co., Ltd., Osaka, Japan).

2.2. Fungal materials and culture conditions

Four strains of *E. nidulans* (NBRC 4342, 5719, 6398, 6577) were provided by the National Institute of Technology and Evaluation, Tokyo, Japan, and other strains were provided by the Medical Mycology Research Center, Chiba University, Japan. All strains were incubated in Czapek-Dox broth (Difco, MD, USA) with 0.5% yeast extract (CDY).

2.3. Reagent materials

Kampo medicines were purchased from TSUMURA Co. (Tokyo, Japan). Crude drugs for decoction were purchased from UCHIDA WAKANYAKU Ltd. (Tokyo, Japan). Sterigmatocystin was purchased Sigma-Aldrich (MO, USA). Aflatoxins B_1 and B_2 were purchased from Makor Chemicals Ltd. (Jerusalem, Israel).

2.4. Preparation of Kampo medicines

Kampo medicines were dissolved at 0.4% in purified water, each solution was centrifuged at 10,700 × *g* for 5 min, and then the supernatant was used as the Kampo solution.

2.5. Preparation of crude drug

Each crude drug (90 g) was extracted under reflux in 1 L purified water for 50 minutes at 100°C, and then filtered through gauze and lyophilized to provide extract powders of the crude drugs.

2.6. Screening of Kampo medicines and crude drugs

Fungi were suspended in sterilized water and inoculated into sterilized CDY containing 0.4% Kampo solution or 0.2% crude drug powder. Aliquots (2 mL, N = 4) were dispended into the wells of a 24 well plate and incubated at 30°C for 7 days, followed by HPLC analysis.

2.7. Sample preprocessing and analytical HPLC conditions

Culture supernatants were lyophilized and extracted with 2 mL MeOH by sonication for 20 min, and then each extract was filtered through cotton and dried. Each dried extract was dissolved in 95% MeOH and analyzed by diode array detector (DAD)-HPLC [mobile phase: A: H_2O, B: MeCN, 30-100% (0-17 min), 100% (17-30 min), column: Mightysil RP-18 column GP II (3.0 × 250 mm, 5 µm), temperature: 40°C, flow rate: 0.5 mL/min, injection volume: 10 µL]. The amount of ST was calculated from the peak area (tR = 16.5 min) on the HPLC chromatogram. All reported values represent the mean ± standard deviation (SD) of at least four experiments.

2.8. Quantification of sterigmatocystin

ST (1 mg) was dissolved in 10 mL CH_3CN and standard solutions (70 to 0.1 µg/mL) were prepared by serial dilution. Each standard solution (10 µL) was analyzed by HPLC by UV at 324 nm and a calibration curve was prepared using a linear approximation for the relationship of area to concentration. The approximate formula was y = 65748x, and the R^2 value was 0.9998.

3. Results and Discussion

3.1. Screening Kampo medicines for the ability to induce fungal metabolite production

E. nidulans IFM 60678 was incubated with 27 Kampo medicines and each culture extract was analyzed by DAD-HPLC (Table 1). The appearance of a new peak (**1**: tR = 16.5 min) was observed in 13 samples (culture extract incubated with Cho-to-san, Hachimi-jio-gan, Hange-koboku-to, Hochu-ekki-to, Juzen-taiho-to, Kakkon-to-ka-senkyu-shini, Kami-shoyo-san, Otsuji-to, Saiko-keishi-to, Shakuyaku-kanzo-to, Shimbu-to, Sho-saiko-to, and Toki-shakuyaku-san) (Fig. 1). Compound **1** was identified as sterigmatocystin (**1**, ST) by comparison

Table 1 Amount of sterigmatocystin (ST) inducted by each Kampo medicine.

Kampo medicine	Sterigmatocystin (µg/mL ± SD)
Bakumondo-to	n. d.
Cho-to-san	11.2 ± 0.5
Daiken-chu-to	n. d.
Dai-saiko-to	n. d.
Gosha-jinki-gan	n. d.
Hachimi-jio-gan	26.5 ± 1.7
Hange-kouboku-to	1.0 ± 0.2
Hange-shashin-to	n. d.
Hochu-ekki-to	1.4 ± 0.2
Juzen-taiho-to	5.4 ± 0.7
Kakkon-to-ka-senkyu-shini	1.2 ± 0.2
Kakkon-to	n. d.
Kami-shoyo-san	0.8 ± 0.1
Keishi-bukuryo-gan	n. d.
Mao-to	tr.
Otsuji-to	22.2 ± 7.8
Ouren-gedoku-to	tr.
Rikkunshi-to	tr.
Ryokei-jutsukan-to	n. d.
Saiboku-to	n. d.
Saiko-keishi-to	2.1 ± 0.6
Sairei-to	tr.
Shakuyaku-kanzo-to	23.5 ± 2.2
Shimbu-to	2.7 ± 0.8
Sho-saiko-to	13.7 ± 3.0
Sho-seiryu-to	n. d.
Toki-shakuyaku-san	6.3 ± 0.3
Control	n. d.

Detection was by UV at 324 nm. Values show the amount of ST per 1 mL of medium and the standard deviation (SD, N = 4). Limit of detection was 10 ng/mL. n. d.: not detected. tr.: trace.

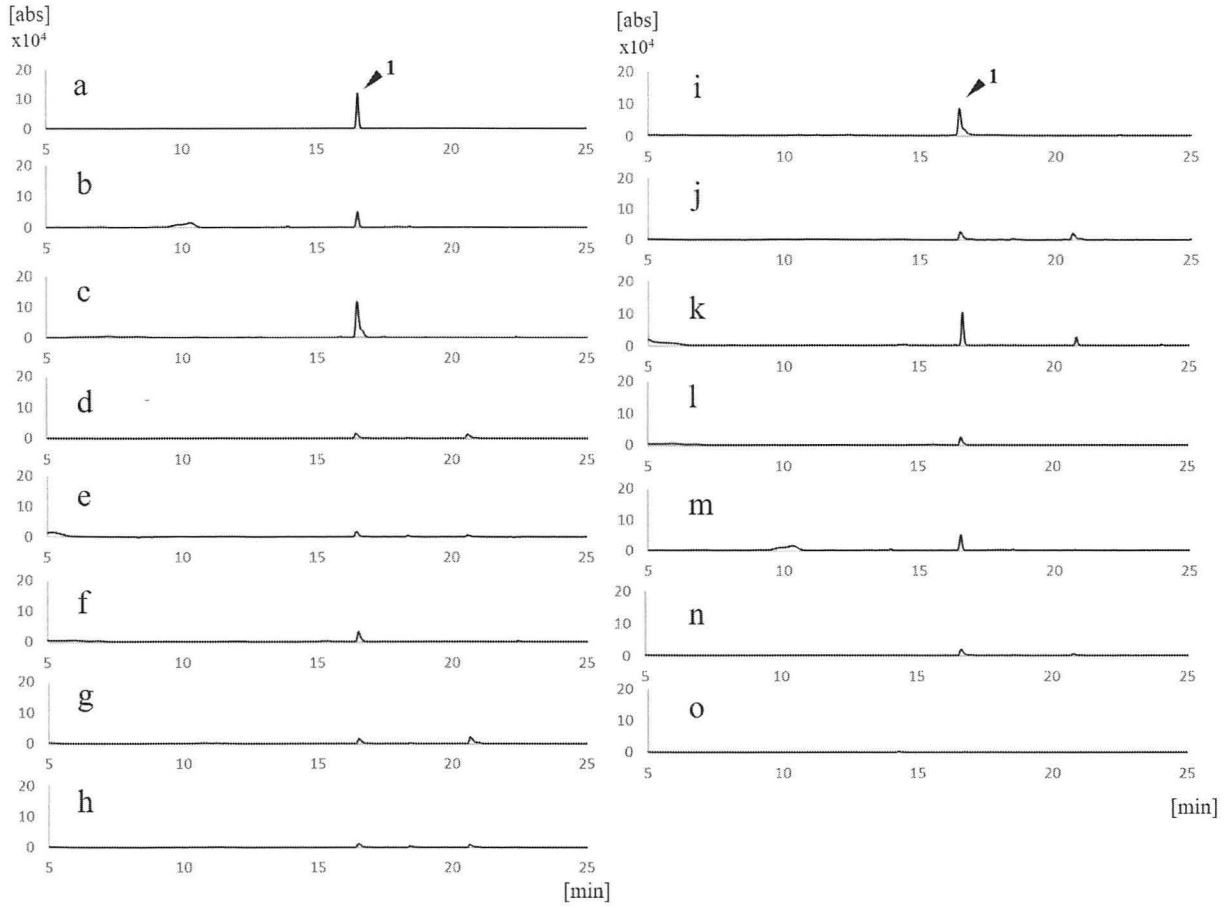

Fig. 1 HPLC chromatograms of sterigmatocystin and culture extracts following culture of *Emericella nidulans* IFM 60678 with several supplements. Detection was by UV at 324 nm. a: sterigmatocystin (standard), b: Cho-to-san, c: Hachimi-jio-gan, d: Hange-koboku-to, e: Hochu-ekki-to, f: Juzen-taiho-to, g: Kakkon-to-ka-senkyu-shini, h: Kami-shoyo-san, i: Otsuji-to, j: Saiko-keishi-to, k: Shakuyaku-kanzo-to, l: Shimbu-to, m: Sho-saiko-to, n: Toki-shakuyaku-san, o: none (control). Sterigmatocystin (**1**): tR = 16.5 min.

of the retention time and UV spectrum on DAD-HPLC analysis with that of the standard (Fig. 2). The production of ST was thus induced by these 13 Kampo medicines (Table 1), and Hachimi-jio-gan (HJG), Otsuji-to (OJT), and Shakuyaku-kanzo-to (SKT) strongly induced ST production. We predicted that a specific component of each Kampo medicine was responsible for inducing ST production and thus we attempted to confirm the ST-inducing activity of Peony root and Glycyrrhiza root (*Glycyrrhiza uralensis*) extracts, two components of SKT, one of the simplest Kampo formulae. Culturing *E. nidulans* IFM 60678 with these crude drug components of SKT showed that Peony root extract has ST-inducing activity (Fig. 3).

3.2. Effect of Peony root on ST production by non-ST-producing *E. nidulans* strains and on Aflatoxins production by *A. flavus* strains

The above results revealed that Peony root extract induces ST production by *E. nidulans* IFM 60678 strain. We examined the background ST production by 39 non-ST-producing *E. nidulans* strains (Table 2) following incubation under the same culture conditions as described above.

Of the 39 strains, 27 did not produce ST and 12 produced ST in the range 0.8 to 21.6 µg/mL. The 27 non-ST-producing strains were incubated with Peony root extract and ST production was observed in 21 strains (78%). The amount of ST produced spanned the range 18.5 µg/mL (IFM 41395) to 1.0 µg/mL (IFM 60678) (Table 3).

We next examined the stimulation of ST production by Peony root in non-ST-producing *A. flavus* strains. *A. flavus* produces aflatoxins B_1 and B_2 via ST. The 22 non-ST-producing *A. flavus* strains shown in supplemental Table 1 were incubated under the same culture conditions as used for *E. nidulans*. None of the *A. flavus* strains produced ST nor aflatoxins upon incubation with Peony root extract.

3.3. The induction of ST production by crude drugs

The results obtained using Peony root extract suggested that crude drugs in Kampo medicines may induce ST production by *E. nidulans*. To investigate this

further, *E. nidulans* IFM 60678 was incubated with 22 crude drugs and each culture extract was analyzed by DAD-HPLC (Table 4). ST production was observed in 11 samples, not observed in 7 samples, and 4 samples inhibited the growth of *E. nidulans*. Of the 11 samples inducing ST production, 4 samples [Alisma tuber (*Alisma orientale*), Bupleurum root (*Bupleurum falcatum*), Japanese Angelica root (*Angelica acutiloba*), and Pinellia tuber (*Pinellia ternate*)] strongly induced ST production (Table 4), of which two (Bupleurum root and Japanese Angelica root) constituting the OJT and Alisma tuber constituting the HJG were included.

Conclusions

In this study, we identified 13 Kampo medicines that induce sterigmatocystin (ST) production by *E. nidulans*. In particular, Hachimi-jio-gan (HJG), Otsuji-to (OJT), and Shakuyaku-kanzo-to (SKT) strongly induced ST production. A further detailed investigation revealed that SKT stimulates ST production through the action of Peony root, a component of SKT. Peony root extract induced ST production in 21 of 27 (78%) non-ST-producing *E. nidulans* strains but did not induce the production of ST or aflatoxins in 22 non-ST-producing *A. flavus* strains. These results suggest that Peony root extract induces ST production by *E. nidulans* specifically.

We also found that 11 crude drugs in Kampo medicines induced ST production by *E. nidulans*, of which 4 (Alisma tuber, Bupleurum root, Japanese Angelica root, and Pinellia tuber) strongly induced ST production.

We previously reported that HJG and OJT strongly induce ST production. However, these Kampo medicines do not contain Peony root. HJG and OJT therefore contain crude components that strongly induce ST production by *E. nidulans*. HJG contains Alisma tuber and OJT contains Bupleurum root and Japanese Angelica root. Therefore, Alisma tuber, Bupleurum root and Japanese Angerica root may contain components that induce ST production by *E. nidulans*.

To our knowledge, our previous and this report are the first to show the induction of fungal secondary metabolites by incubation with Kampo medicines. We think that this induction is unique and that this facile approach may be useful for discovering new natural products and for controlling the production of mycotoxins in the near future.

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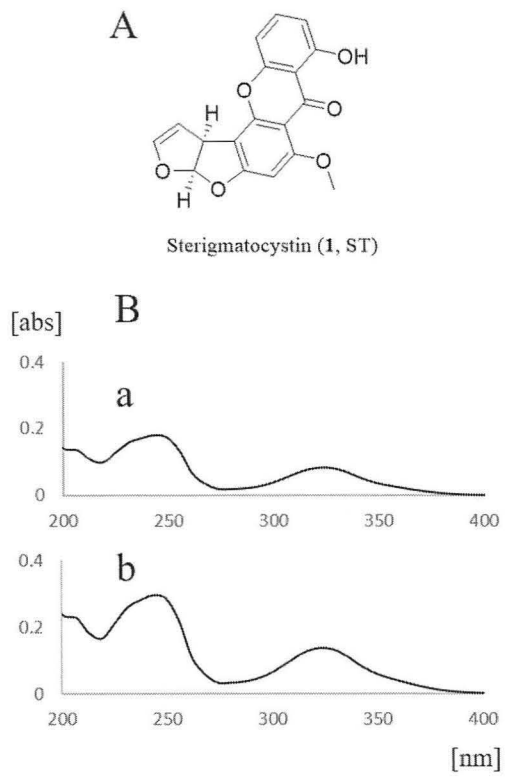

Fig. 2 Chemical structure and UV spectrum of sterigmatocystin. A: chemical structure. B: UV spectrum of standard (a) and peak 1 (b).

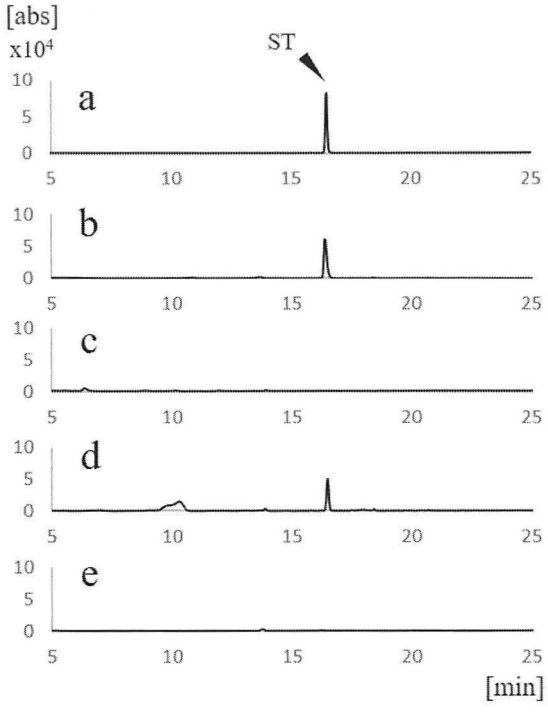

Fig. 3 HPLC chromatograms of sterigmatocystin and culture extracts following culture of *Emericella nidulans* IFM 60678 with several supplements. Detection was by UV at 324 nm. a: sterigmatocystin (standard), b: Peony root, c: Glycyrrhiza root, d: Shakuyaku-kanzo-to, e: none (control). Sterigmatocystin (ST): tR = 16.5 min.

Table 2 Amount of sterigmatocystin (ST) produced by several fungal strains growing in non-supplemented medium.

Fungi	Strain	Source	Sterigmatocystin (μg/mL ± SD)
Emericella nidulans	IFM 5369	unknown	n. d.
Emericella nidulans	IFM 40838	unknown	n. d.
Emericella nidulans	IFM 41094	unknown	7.7 ± 0.7
Emericella nidulans	IFM 41395	unknown	n. d.
Emericella nidulans	IFM 41396	patient	n. d.
Emericella nidulans var. *lata*	IFM 42011	unknown	4.9 ± 0.5
Emericella nidulans var. *acristata*	IFM 42016	exposed fabric	n. d.
Emericella nidulans	IFM 42018	subramanian	n. d.
Emericella nidulans var. *dentata*	IFM 42028	finger nail of man	n. d.
Emericella nidulans var. *acristata*	IFM 42030	soil	n. d.
Emericella nidulans var. *dentata*	IFM 42044	herbal drug	1.2 ± 0.4
Emericella nidulans	IFM 42319	unknown	n. d.
Emericella nidulans	IFM 46997	unknown	n. d.
Emericella nidulans	IFM 46999	unknown	n. d.
Emericella nidulans	IFM 47000	unknown	n. d.
Emericella nidulans	IFM 47001	unknown	n. d.
Emericella nidulans	IFM 47002	unknown	n. d.
Emericella nidulans	IFM 47003	river sediment	n. d.
Emericella nidulans	IFM 47004	soil	5.7 ± 1.2
Emericella nidulans	IFM 47005	soil	n. d.
Emericella nidulans	IFM 47006	paddy field soil	1.0 ± 0.2
Emericella nidulans	IFM 47793	unknown	n. d.
Emericella nidulans	IFM 51356	Bronchial lavage fluid	16.4 ± 1.2
Emericella nidulans	IFM 52249	soil	n. d.
Emericella nidulans	IFM 52250	soil	n. d.
Emericella nidulans	IFM 54308	soil	12.7 ± 0.7
Aspergillus nidulans	IFM 56365	oral	n. d.
Emericella nidulans	IFM 57839	unknown	n. d.
Aspergillus nidulans	IFM 57842	soil	6.2 ± 1.3
Aspergillus nidulans	IFM 59750	air sampling	n. d.
Aspergillus nidulans	IFM 60678	unknown	n. d.
Emericella nidulans var. *echinulata*	IFM 61956	sputum	0.8 ± 0.1
Emericella nidulans	IFM 62671	Bronchial lavage fluid	n. d.
Aspergillus nidulans	IFM 63297	Bronchial lavage fluid	4.3 ± 1.0
Emericella nidulans var. *echinulata*	IFM 64750	Bronchial lavage fluid	21.6 ± 1.1
Emericella nidulans	NBRC 4342	kaoliangchiu yeast cake	n. d.
Emericella nidulans	NBRC 5719	unknown	3.6 ± 0.7
Emericella nidulans	NBRC 6398	unknown	n. d.
Emericella nidulans	NBRC 6577	unknown	n. d.

Detection was by UV at 324 nm. Values show the amount of ST per 1 mL of medium and the standard deviation (SD, N = 4).
Limit of detection was 10 ng/mL. n. d.: not detected.

Table 3 Amount of sterigmatocystin (ST) produced by several fungal strains following induction by Peony root extract.

Fungi[a]	Strain	Source	Sterigmatocystin (μg/mL ± SD)
Emericella nidulans	IFM 5369	unknown	5.3 ± 0.4
Emericella nidulans	IFM 40838	unknown	4.2 ± 0.4
Emericella nidulans	IFM 41395	unknown	18.5 ± 1.9
Emericella nidulans	IFM 41396	patient	n. d.
Emericella nidulans var. *acristata*	IFM 42016	exposed fabric	3.7 ± 1.2
Emericella nidulans	IFM 42018	subramanian	n. d.
Emericella nidulans var. *dentata*	IFM 42028	finger nail of man	1.2 ± 0.2
Emericella nidulans var. *acristata*	IFM 42030	soil	1.7 ± 0.4
Emericella nidulans	IFM 42319	unknown	n. d.
Emericella nidulans	IFM 46997	unknown	18.4± 0.8
Emericella nidulans	IFM 46999	unknown	n. d.
Emericella nidulans	IFM 47000	unknown	9.1 ± 1.8
Emericella nidulans	IFM 47001	unknown	8.5 ± 1.2
Emericella nidulans	IFM 47002	unknown	3.4 ± 0.2
Emericella nidulans	IFM 47003	river sediment	4.0 ± 0.2
Emericella nidulans	IFM 47005	soil	1.6 ± 1.1
Emericella nidulans	IFM 47793	unknown	2.1 ± 0.1
Emericella nidulans	IFM 52249	soil	1.7 ± 0.7
Emericella nidulans	IFM 52250	soil	5.9 ± 1.5
Aspergillus nidulans	IFM 56365	oral	n. d.
Emericella nidulans	IFM 57839	unknown	15.6 ± 1.0
Aspergillus nidulans	IFM 59750	air sampling	n. d.
Aspergillus nidulans	IFM 60678	unknown	1.0 ± 0.2
Emericella nidulans	IFM 62671	Bronchial lavage fluid	8.1 ± 0.4
Emericella nidulans	NBRC 4342	kaoliangchiu yeast cake	15.0 ± 1.2
Emericella nidulans	NBRC 6398	unknown	6.4 ± 0.3
Emericella nidulans	NBRC 6577	unknown	8.7 ± 0.6

Detection was by UV at 324 nm. Values show the amount of ST per 1 mL of medium and the standard deviation (SD, N = 4).
Limit of detection was 10 ng/mL. n. d.: not detected. a: non-ST-producing strain without Peony root extract.

Table 4 Amount of sterigmatocystin (ST) inducted by several crude drugs in Kampo medicines.

Crude drug	Scientific name	Sterigmatocystin (μg/mL ± SD)
Alisma tuber	*Alisma orientale*	46.8 ± 3.7
Atractylodes Lancea rhizome	*Atractylodes lancea*	3.4 ± 0.5
Bupleurum root	*Bupleurum falcatum*	29.4 ± 9.4
Cinnamon bark	*Cinnamomum cassia*	n. d.
Citrus Unshiu peel	*Citrus unshiu*	n. d.
Coptis rhizome	*Coptis japonica*	n. g.
Ephedra herb	*Ephedra sinica*	n. d.
Ginger	*Zingiber officinale*	2.1 ± 0.4
Immature orange	*Citrus aurantium*	n. d.
Japanese Angelica root	*Angelica acutiloba*	44.5 ± 5.9
Jujube	*Zizyphus jujuba*	n. d.
Magnolia bark	*Magnolia obovata*	n. g.
Magnolia flower	*Magnolia kobus*	n. g.
Moutan bark	*Paeonia suffruticosa*	n. d.
Panax Japonicus rhizome	*Panax japonicus*	1.2 ± 0.8
Perilla herb	*Perilla frutescens*	9.6 ± 0.8
Phellodendron bark	*Phellodendron amurense*	n. g.
Pinellia tuber	*Pinellia ternata*	27.3 ± 11.6
Poria sclerotium	*Wolfiporia cocos*	4.2 ± 0.4
Processed Aconite root	*Aconitum carmichaeli*	2.4 ± 0.2
Rehmannia root	*Rehmannia glutinosa*	2.4 ± 0.4
Scutellaria root	*Scutellaria baicalensis*	n. d.

Detection was at UV 324 nm. Values show the amount of ST per 1 mL of medium and the standard deviation (SD, N = 4).
Limit of detection was 10 ng/mL. n. d.: not detected. n. g.: no growth.

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芍薬甘草湯及び他の漢方処方の構成生薬が*Emericella nidulans*のsterigmatocystin産生に与える効果

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漢方薬が真菌の二次代謝産物産生能に与える影響を調べるため，漢方薬添加培地で*Emericella nidulans* IFM 60678を培養した結果，芍薬甘草湯を含む十数種類の漢方薬でsterigmatocystin（ST）が産生された．また，芍薬甘草湯の構成生薬である芍薬エキスがST誘導能を示すことを明らかとした．次に，非ST産生*E. nidulans*および*Aspergillus flavus*菌株に対する芍薬エキスの作用について検討し，その作用が*E. nidulans*のみに有効である可能性を見出した．さらに他の生薬について*E. nidulans*に対するST誘導能を検討した結果，芍薬以外の生薬も*E. nidulans*のST産生を誘導する作用があることが，明らかとなった．我々はこのユニークでシンプルな方法が，新規天然物の発見やマイコトキシンの産生制御に繋がると考えている．

キーワード：漢方薬；芍薬；芍薬甘草湯；生薬；*Emericella nidulans*；sterigmatocystin