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Inhibitory Effects of Spices on Growth and Toxin Production of Toxigenic Fungi

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The inhibitory effects of 29 commercial powdered spices on the growth and toxin production of three species of toxigenic *Aspergillus* were observed by introducing these materials into culture media for mycotoxin production. Of the 29 samples tested, cloves, star anise seeds, and allspice completely inhibited the fungal growth, whereas most of the others inhibited only the toxin production. Eugenol extracted from cloves and thymol from thyme caused complete inhibition of the growth of both *Aspergillus flavus* and *Aspergillus versicolor* at 0.4 mg/ml or less. At a concentration of 2 mg/ml, anethol extracted from star anise seeds inhibited the growth of all the strains.

In experiments with naturally occurring mycotoxins in certain foodstuffs, we (6-8) have already demonstrated that cinnamon and pepper contain inhibitory materials and that cinnamon completely inhibits the growth of toxigenic *Aspergillus* strains, whereas other herbal drugs and commercial dry condiments tested inhibit only the toxin production. On the basis of our studies, Morozumi (12) carried out a chemical study on the inhibitor of cinnamon and identified it as *o*-methoxycinnamaldehyde. He also pointed out that this substance has a strong inhibitory effect on the growth of dermatophytes such as *Microsporum*, *Trichophyton*, and *Epidermophyton*.

This paper deals with the inhibitory effects of various kinds of commercial spices on the growth and toxin production of common toxigenic fungal strains as part of a search for substances with cinnamon-like inhibitory effects.

MATERIALS AND METHODS

Samples. The commercial and scientific names of spices tested are as follows: cloves (*Caryophyllus*), star anise seeds (*Illicium anisatum*), allspice (*Pimenta*), horseradish (*Armoracium*), thyme (*Thymus*), caraway seeds (*Carum carvi*), celery leaves (*Apium graveolens*), sage leaves (*Salvia*), estragon leaves (*Artemesium*), dillseeds (*Anethum*), turmeric (*Curcumae domesticae*), marjoram leaves (*Origanum majorana*), basil leaves (*Ocimum basilicum*), anise seeds (*Pimpinella anisum*), cumin fruits (*Cuminum*), coriander seeds (*Coriandrum sativum*), fennel seeds (*Foeniculum*), mace (*Myristica arillus*), laurel leaves (*Laurus*), mint leaves (*Mentha piperita*), oregano leaves (*Origanum*), rosemary leaves (*Rosmarinus*), nutmeg seeds (*Myristica*), poppy seeds (*Papaver*), paprika seeds (*Capsicum*), savory leaves (*Calamintha*), cardamom fruits (*Elettaria cardamomom*), ginger (*Zingiber officinale*), and cayenne pepper seeds (*Capsicum*).

All of these materials were purchased as groceries in June 1977. All samples used in the experiments were ground to a powder before use.

The procedure for extraction, the organisms, the inoculation procedure, and the mycotoxin analysis were as described previously (8).

Extraction. Eugenol, anethol, and thymol used in these experiments were extracted from powdered cloves, powdered star anise seeds, and powdered thyme, respectively. The extractions were carried out by the methods described in the Japanese Pharmacopeia IX (25). The extracts were obtained in the following ways. A 100-g powdered sample was steam distilled with 500 ml of distilled water at 100°C for 8 h in a 1,000-ml Erlenmeyer flask. The essential oil in the evaporated fraction was collected in 10 ml of *n*-hexane, and the *n*-hexane layer was concentrated by evaporation. Further purification of the fraction was done by thin-layer chromatography, using Kiesel gel GF_{254} (0.25 mm, E. Merck AG, Darmstadt, Germany) plates. For chromatographic analysis of the each extract, developing solvents were employed as follows: petroleum ether-diethyl ether (7:3, vol/vol) for eugenol, chloroform-benzene (3:1) for thymol, and benzene for anethol, by the methods of Stahl (26). These substances were found as dark spots on thin-layer chromatography plates under ultraviolet light (model UV-LS; wavelength, 235 nm; Irie Ltd., Tokyo) at R_f 0.7 (anethol) and at R_f 0.5 (eugenol and thymol). The visualized spots were recovered from the thin-layer chromatography plate, using a thin-layer chromatography fractionator (Toyo Kagaku Sangyo Co., Tokyo), with 10 ml of ethanol. Each isolated fraction was compared with commercial standard samples (E. Merck AG) by gas-liquid chromatography by using a Hitachi 163 chromatograph (Hitachi Ltd., Tokyo) under the following experimental conditions: column, 2

m by 3 mm (inside diameter), packed with 5% Xe-60 Chromosorb-W-AW; column temperature, 150°C; carrier gas, N_2; flow rate, 25 ml/min; detector, flame ionization detector. These three fractions were identified by means of gas chromatography-mass spectrometry by the method described by Masada (11). The instrument used was the Hitachi RMU-6MG (Hitachi Ltd., Tokyo) with experimental conditions as follows: column, 1 m by 3 mm (inside diameter), glass column packed with 5% Dexsil-Chromosorb-W-AW; column temperature, 150 to 250°C; carrier gas, He; column pressure, 1.33 kg/cm; detector, TIM; ionization voltage, 70 eV.

Organisms. The fungal strains used in this study were the following: an aflatoxin-producing strain of *Aspergillus flavus* ATCC 28539, an ochratoxin A-producing strain of *Aspergillus ochraceus* 10-21-N originally isolated from the atmosphere, and a sterigmatocystin-producing strain of *Aspergillus versicolor* I-20-b isolated from small dried sardines. All cultures were maintained on potato-dextrose agar slants (PDA; Eiken Chemical Co., Ltd., Tokyo).

Inoculation. The strains were cultivated on PDA slants for about 10 days at 25°C until they were well sporulated, and then the spores were harvested in 1% Driwel (0.1% polyethylene glycol sorbitan monooleate, Fuji Photo Film Co., Ltd., Tokyo). The spore suspension thus obtained was further adjusted with sterile 1% Driwel to give a final spore concentration of approximately 10^4 spores per ml. A 0.1-ml portion of this suspension was inoculated into 9 ml of culture medium containing either 1 g of the powdered sample or a suitable volume of diluted extract. The culture media used were low-salt broth (SL, 22) to produce aflatoxin, and yeast extract-sucrose broth (YES, 3) to produce ochratoxin A and sterigmatocystin.

The tube cultures were slanted and incubated without shaking at 25°C for 8 days in the case of the aflatoxin-producing and ochratoxin A-producing strains and for 12 days in the case of the sterigmatocystin-producing strain.

Analysis. Mold growth was observed visually throughout the incubation period. After the incubation, the cultures were autoclaved (121°C for 30 min) to kill the spores and the vegetative mycelia. The dry weights of mycelial mat were determined at the end of the incubation period as follows. The culture was filtered through a glass filter (Shibata Chemical Apparatus Manufacturing Co., Ltd., Tokyo). The fungal mat was dried at 50°C for 48 h and then stored over silica gel for 1 week. The weight of the dried fungal mat was then determined.

Cultures were then assayed for mycotoxin production as follows. Ten milliliters of chloroform was added to the same volume of the culture fluid in a test tube, and the tube was left for approximately 5 min in a water bath maintained at 40°C. The chloroform fraction was passed through anhydrous sodium sulfate in a funnel and then concentrated to 0.5 ml in a flash evaporator. Mycotoxin assays were then carried out by thin-layer chromatography, using Adsorbosil-1 plates developed with chloroform-acetone (9:1) for aflatoxins, with benzene-acetic acid (9:1) for ochratoxin A, and with benzene-methanol-acetic acid (90:1:5) for sterigmatocystin. The intensities of fluorescence of the separated mycotoxin spots were then measured with a fluorodensitometer (model MPF-2A, Hitachi Ltd., Tokyo).

Each test culture was compared with an uncontrolled culture, which showed uninhibited growth of the inoculated fungus.

RESULTS

Inhibitory effects of spices on the growth and toxin production of toxigenic fungi. The inhibitory effects of the 29 kinds of powdered spices were tested. Three of the spices, namely, cloves, star anise seeds, and allspice, caused complete inhibition of the growth of the three toxigenic *Aspergillus* strains. These three spices showed inhibitory effects similar to that of cinnamon powder (8).

Horseradish completely inhibited the growth of *A. flavus*, partially (66%) inhibited the growth and completely inhibited the toxin production of *A. versicolor*, and partially inhibited (20 and 46%) the growth and toxin production of *A. ochraceus*. Thyme caused 10 to 90% inhibition of the growth of these three *Aspergillus* strains, but showed nearly complete inhibition (from 86 to 100%) of their toxin production. Caraway seeds, celery leaves, and sage leaves caused partial (from 0 to 88%) inhibition of the growth and toxin production of both *A. ochraceus* and *A. versicolor*, although caraway seeds caused complete inhibition of ochratoxin production of *A. ochraceus*. Estragon leaves produced complete inhibition of sterigmatocystin production by *A. versicolor*, but only partially inhibited the growth of the three *Aspergillus* strains and toxin production by *A. flavus* and *A. ochraceus*.

Eight substrates, namely, dill seeds, turmeric, marjoram, basil leaves, anise seeds, cumin fruits, and coriander seeds, showed complete inhibition of ochratoxin A production of *A. ochraceus* and partial inhibition of the growth and toxin production of *A. flavus* and *A. versicolor*, and of the growth of *A. ochraceus*.

The other 12 spices had relatively minor effects (from 0 to 41%) on the growth and toxin production of all three *Aspergillus* strains.

Inhibitory effects of the end products of eugenol, anethol, and thymol on the growth and toxin production of toxigenic fungi. In these experiments, the recoveries of the essential oils extracted from powdered spices were as follows: 15.4% in cloves, 4.9% in star anise seeds, and 0.59% in thyme, respectively. The essential oil fraction of cloves contained 92% eugenol, that of star anise seeds contained 89% anethol, and that of thyme contained 54% thymol. The results of the chromatograms on gas-liquid chromatog-

raphy analysis showed that all of the end products used in these experiments were over 98% pure.

Eugenol extracted from powdered cloves (Table 1) completely inhibited the growth of both *A. flavus* and *A. versicolor* at a concentration of 250 μg/ml and partially inhibited (83%) the ochratoxin A production of *A. ochraceus*. At a concentration of 125 μg/ml, eugenol caused partial inhibition (40%) of the growth of *A. flavus* and complete inhibition of its aflatoxin production, slight inhibition (32%) of the growth of *A. ochraceus* and partial inhibition (73%) of its ochratoxin A production, and negligible inhibition (27%) of the growth of *A. versicolor* with 95% inhibition of its sterigmatocystin production. Even at a concentration of 31.2 μg/ml, eugenol showed some effects.

Anethol extracted from powdered star anise seeds completely inhibited the growth of the three *Aspergillus* strains at a concentration of 2 mg/ml (Table 1). At a concentration of 1 mg/ml, anethol did not effectively inhibit the growth and toxin production of *A. flavus* or the growth of *A. ochraceus* (Table 2). At the same concentration, anethol showed slight inhibition of the growth of *A. ochraceus* and partial inhibition (75%) of ochratoxin A production by the fungus. In regard to the growth and sterigmatocystin production of *A. versicolor*, anethol showed partial (57%) and marked 93%) inhibition, respectively, at the same concentration. Even at lower concentrations, it was clear that the effective inhibition of the growth and toxin production of both *A. ochraceus* and *A. versicolor* was maintained.

Thymol extracted from powdered thyme completely inhibited the growth of the three *Aspergillus* strains at a concentration of 400 μg/ml, as shown in Table 3. At a concentration of 200 μg/ml, thymol showed partial (72%) and almost complete (98%) inhibition, respectively, of the growth and aflatoxin production of *A. flavus*. At the same concentration, thymol effectively (96 and 100%) inhibited both the growth and ochratoxin A production of *A. ochraceus* and partially inhibited (58%) the growth of *A. ochraceus*, but had no effect on its ochratoxin A production at a concentration of 100 μg/ml. At the same concentration, thymol showed partial (61%) and nearly complete (97%) inhibition, respectively, of the growth and sterigmatocystin production of *A. versicolor*. Below 100 μg/ml, this substance partially inhibited (34 and 80%) the growth and sterigmatocystin production of *A. versicolor*, but had little effect on the growth and toxin production of both *A. flavus* and *A. ochraceus* (Table 3).

DISCUSSION

It has been known for some time that certain crude drugs and spices contain substances with

TABLE 1. *Inhibitory effects of eugenol on the growth and toxin production of toxigenic fungi in an appropriate broth for toxin production*

Level of eugenol (μg/ml)	*A. flavus*		*A. ochraceus*		*A. versicolor*	
	Mycelia (mg)	Aflatoxin B_1 (μg/ml)	Mycelia (mg)	Ochratoxin A (μg/ml)	Mycelia (mg)	Sterigmatocystin (μg/ml)
500	0 (100)[a]	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
250	0 (100)	0 (100)	6 (98)	0.5 (83)	0 (100)	0 (100)
125	110 (40)	0 (100)	248 (32)	0.7 (76)	427 (27)	0.8 (95)
62.5	148 (19)	2.5 (83)	335 (8)	3.0 (0)	555 (5)	4.5 (70)
31.2	182 (14)	3.5 (76)	399 (0)	2.5 (17)	563 (4)	6.0 (60)
Control	183	15.0	365	3.0	585	15.0

[a] Numbers in parentheses indicate percent inhibition.

TABLE 2. *Inhibitory effects of anethol on the growth and toxin production of toxigenic fungi in an appropriate broth for toxin production*

Level of anethol (mg/ml)	*A. flavus*		*A. ochraceus*		*A. versicolor*	
	Mycelia (mg)	Aflatoxin B_1 (μg/ml)	Mycelia (mg)	Ochratoxin A (μg/ml)	Mycelia (mg)	Sterigmatocystin (μg/ml)
2	0 (100)[a]	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
1	270 (0)	15.0 (0)	439 (0)	0.8 (73)	253 (57)	1.0 (93)
0.5	246 (0)	15.0 (0)	614 (0)	1.0 (67)	426 (28)	6.0 (60)
0.25	258 (0)	15.0 (0)	607 (0)	1.5 (50)	511 (13)	15.0 (0)
Control	183	15.0	365	3.0	585	15.0

[a] Numbers in parentheses indicate percent inhibition.

TABLE 3. *Inhibitory effects of thymol on the growth and toxin production of toxigenic fungi in an appropriate broth for toxin production*

Level of thymol (μg/ml)	*A. flavus*		*A. ochraceus*		*A. versicolor*	
	Mycelia (mg)	Aflatoxin B_1 (μg/ml)	Mycelia (mg)	Ochratoxin A (μg/ml)	Mycelia (mg)	Sterigmatocystin (μg/ml)
400	0 (100)[a]	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
200	51 (72)	0.3 (98)	12 (96)	0 (100)	0 (100)	0 (100)
100	138 (25)	15.0 (0)	155 (58)	5.0 (0)	230 (61)	0.5 (97)
50	237 (0)	15.0 (0)	414 (0)	5.0 (0)	385 (34)	2.5 (83)
25	NT[b]	NT	NT	NT	597 (0)	15.0 (0)
Control	183	15.0	365	3.0	585	15.0

[a] Numbers in parentheses indicate percent inhibition.
[b] NT, Not tested.

antifungal activity in their essential oils (1, 2, 4, 5, 9, 10, 13–20).

Quite recently, the authors (8) studied various kinds of herbal drugs and commercial dry condiments and showed that these samples inhibited the growth and toxin production of toxigenic fungi. Scott and Kennedy (23, 24) pointed out that piper nigrum prevented aflatoxin production by *A. flavus*. About the problem, the authors (6) also noted that chloroform extracts of cinnamon and pepper seeds inhibited the growth of toxigenic fungi such as *A. flavus*, *A. parasiticus*, *A. ochraceus*, *A. versicolor*, and *Fusarium solani*.

As described above, powdered cloves, star anise seeds, and allspice acted as growth inhibitors toward the three toxigenic *Aspergillus* species.

Eugenol, extracted from cloves, showed clear inhibition of the growth of *A. flavus* and *A. versicolor* at a concentration of 250 μg/ml. Anethol, extracted from star anise seeds, inhibited the growth of the three toxigenic *Aspergillus* strains at a concentration of 2 mg/ml. Thymol, extracted from thyme, completely inhibited the growth of *A. flavus* at a concentration of 400 μg/ml and completely inhibited the toxin production of *A. ochraceus* and the growth of *A. versicolor* at a concentration of 200 μg/ml.

The essential oil fractions of cloves and allspice contain from 70 to 95% eugenol, that of star anise seeds contains from 85 to 90% anethol, and that of thyme contains from 20 to 60% thymol (11, 21, 26).

The results of these experiments indicate that the essential oil fraction of cloves contained 92% eugenol, that of star anise seeds contained 89% anethol, and that of thyme contained 54% thymol. These three end products were regarded to be pure over 98%. The results, calculated on the basis of the above contents, were: each 1 g of powdered spice sample contained 142 mg of eugenol (in cloves), 44 mg of anethol (in allspice), and 3 mg of thymol (in thyme), respectively. Eugenol and thymol showed an almost equal inhibitory effect (minimum inhibitory concentration, 500 and 400 μg/ml) and showed stronger effects than anethol (minimum inhibitory concentration, 2 mg/ml). It is assumed that each of the three powdered spices was able to inhibit completely the growth of the three *Aspergillus* strains at a concentration of 3.5 mg of cloves, of 45.5 mg of allspice, and of 133.4 mg of thyme in 1 ml of the medium. Therefore, the powdered cloves, of 29 kinds of powdered sample tested, has the strongest inhibitory effect on the growth of the fungi.

The antifungal effects of the three end products demonstrated fungal growth inhibition of the strains at high concentrations and inhibition of toxin production of the strains at lower concentrations. These results clearly show that the inhibitory effects of these powdered spices are largely accounted for by the main component of the essential oils in each powdered sample.

On the other hand, powdered horseradish also showed inhibitory effects on the growth and toxin product of toxigenic fungi, although there is no evidence in the literature that allyl isothianate, the major component in the essential oil of horseradish, has such inhibitory effects. Similarly, the essential oils of powdered estragon, tumeric, cumin fruits, and coriander seeds contain about 65% estragole (11, 21), about 60% turmerone (26), from 35 to 62% cuminaldehyde (11, 21), and from 60 to 70% linalool (11, 21), respectively, but the inhibitory effects of these compounds have not been investigated.

Thus, it seems likely that the inhibitory effects of cloves, star anise seeds, thyme, and possibly other spices are due to the major component in the essential oils of the powdered samples. These inhibitory effects are interesting in connection with the prevention of mycotoxin contamination in many foods, and these compounds represent possible alternatives to the food additives in use at present.

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