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Isolation, Purification, and Antibiotic Activity of o-Methoxycinnamaldehyde from Cinnamon

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o-Methoxycinnamaldehyde has been isolated and purified from powdered cinnamon. The compound inhibits the growth and toxin production of mycotoxin-producing fungi. The substance completely inhibited the growth of *Aspergillus parasiticus* and *A. flavus* at 100 μg/ml and *A. ochraceus* and *A. versicolor* at 200 μg/ml. It inhibited the production of aflatoxin B_1 by over 90% at 6.25 μg/ml, ochratoxin A at 25 μg/ml, and sterigmatocystin at 50 μg/ml. The substance also displayed a strong inhibitory effect on the growth of five dermatophytoses species, e.g., *Microsporum canis* (minimum inhibitory concentration, 3.12 to 6.25 μg/ml). However, no antibacterial effect was observed at concentrations as high as 50 μg/ml.

Antimicrobial substances contained in common spices have been intensively investigated (2-4, 11, 13-17). Okazaki et al. (19-42) extensively studied the microbial effects of over 1,300 species of higher plants in Japan, including herbal drugs, and 50 kinds of essential oils. The authors concluded that cinnamaldehyde (CA) exhibited the highest antifungal properties.

Frazier (7) has suggested that spices appear to be useful antimicrobial agents in preventing growth of undersirable organisms in food.

Hartung et al. (8) reported a decrease in the growth of *Aspergillus parasiticus* and aflatoxin production in raisin bread containing cinnamon when compared with breads not containing cinnamon.

Additionally, Bullerman (3) indicates that cinnamon in concentration as low as 0.02% inhibited mold growth and aflatoxin production in yeast extract sucrose broth. Hitokoto et al. (9) also reported that cinnamon inhibited growth of *A. parasiticus, A. versicolor, A. ochraceus,* and *Fusarium solani.*

Cinnamon contains 0.5 to 1.0% volatile oil composed mainly of CA (65 to 75%), eugenol (4 to 10%), cinnamic acid, and *o*-methoxycinnamaldehyde (OMCA) (10, 44-46). Bullerman et al. (4) studied the inhibition of fungal growth and aflatoxin production by CA and eugenol and concluded that these compounds are the major antifungal substances of cinnamon oil.

The object of this study was to elucidate the properties of another antimicrobial substance —OMCA—isolated and purified from cinnamon, as well as its inhibitory effect on fungal growth and mycotoxin production.

MATERIALS AND METHODS

Powdered cinnamon. Powdered cinnamon (Cinamoni Cortex Pulveratus; Nippon Funmatsu Yakuhin & Co., Osaka, Japan) was commercially prepared in accordance with the Japanese pharmacopeia (47).

Isolation and purification of antifungal substance isolated from cinnamon (OMCA). The purification scheme is shown in Fig. 1. Powdered cinnamon weighing 1 kg was steeped in 5 liters of chloroform at room temperature and allowed to stand for 2 h. The mixture was evaporated to dryness on a rotary evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to yield 25.3 g of residue. A 20-g quantity of the residue was dissolved in 100 ml of *n*-hexane, the resulting *n*-hexane soluble fraction was then applied to a kieselgel 60 (70-230 mesh; E. Merck AG, Darmstadt, Germany) chromatographic column. The column (2 by 20 cm) was eluted with 150 ml each of *n*-hexane, benzene:chloroform (9:1), and chloroform. The benzene:chloroform fraction exhibiting fungal growth inhibition, was concentrated, redissolved in 10 ml of benzene, and applied to an aluminum oxide (90 active, neutral; E. Merck AG) chromatographic column. The column (2 by 16 cm) was washed first with 150 ml each of benzene and benzene:chloroform (9:1), and then eluted with 150 ml of chloroform. The chloroform eluate was vacuum evaporated to 10 ml. One of the chloroform concentrates was applied to preparative thin-layer chromatography (PTLC) with a kieselgel GF-254 plate (20 by 20 cm; E. Merck AG) and developed with benzene:acetone (19:1) at room temperature. After development, the plate was examined under UV light at 365 and 254 nm. The visualized spots were recovered from the TLC plate using a TLC fractionator (Toyo Kagaku Sangyo Co., Tokyo, Japan). Each TLC fraction was eluted with 10 ml of ethanol to yield six fractions. The T-4 fraction (exhibiting fungal growth inhibition) was reapplied to a PTLC plate to yield three fractions. Each fraction was analyzed with a gas

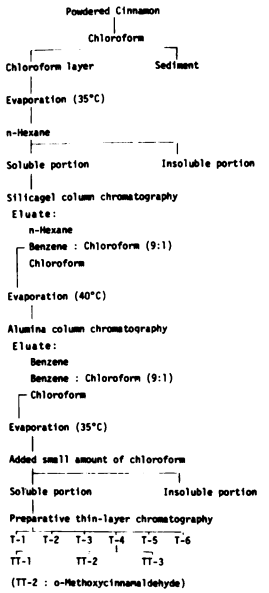

FIG. 1. *Isolation of the antifungal substance from cinnamon.*

chromatograph-mass spectrometer and TLC.

Gas chromatography-mass spectrometry. The instrumentation used was the Hitachi RMU-6MG (Hitachi Ltd., Tokyo, Japan) with experimental conditions as follows: column, 1 m by 3 mm (ID), 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.

Reagents. CA, cinnamyl alcohol, cinnamic acid, and eugenol were obtained from Wako Pure Chemicals Inc., Osaka, Japan. OMCA was synthesized from *o*-methoxybenzaldehyde and acetaldehyde as described by Bertram and Kursten (1).

Inhibitory effect of each fraction against mycotoxin-producing fungi. (i) Microorganisms. Four mycotoxin-producing fungi were used for antifungal evaluation: *A. parasiticus* NRRL-3000 and *A. flavus* ATCC-28539 (aflatoxin); *A. ochraceus* stA-15, originally isolated from the atmosphere (ochratoxin); and *A. versicolor* I-20-b isolated from dried small sardine (sterigmatocystin). Only *A. flavus* was used for the antifungal properties evaluation during the purification steps; however, all strains were used for the antifungal studies of the chloroform extract and the final product.

(ii) Fractions. The chloroform concentrate and dried, purified final product were diluted or dissolved (wt/vol) in methanol as applicable to make 10-fold or 2-fold dilutions, respectively. A 100-μl sample of each diluted solution was added to separate test tubes containing 10 ml of each broth. Each fraction obtained during the purification process was dissolved in 100 μg of methanol and added to separate test tubes. The test tubes were sterilized at 110°C for 10 min before inoculation with the fungal spore suspensions.

(iii) Determination of antifungal activity. The antifungal and toxin production properties of each fraction were evaluated with the following media: low salt (SL) broth (43) and yeast extract-sucrose (YES) broth (5). The inoculum was prepared by growing the organism on potato dextrose agar (Eiken Chemical Co., Ltd., Tokyo, Japan) slants for approximately 7 days at 25°C. The spores were harvested by adding 10 ml of sterilized 0.1% Driwel (polyethylene glycol sorbitan monooleate; Fuji Photo Film Co., Ltd., Tokyo, Japan) solution and aseptically dislodging the spores with a sterile inoculating loop. The solution was filtered on a membrane filter (pore size, 8.0 μm; Nihon Millipore Ltd., Tokyo, Japan). The spore suspension thus obtained was further adjusted with sterile Driwel solution to give a final concentration of approximately 10^4 spores per ml. The inoculum was 10^3 spores per ml (0.1 ml of the suspension). The cultures were incubated at 25°C in a slant for 8 days for aflatoxin-producing strains and for the ochratoxin-producing strain and 12 days for the sterigmatocystin-producing strain.

After the incubation, the culture was filtered through a glass filter (Shibata Chemical Apparatus Manufacturing Co., Ltd., Tokyo, Japan). 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.

Mycotoxin production was evaluated by the methods of Eppley (6) and Naoi et al. (18). The mycotoxin was extracted by adding 10 ml chloroform to the cunture and holding in a water bath at 40°C. The extract was passed through anhydrous sodium sulfate and concentrated at 0.5 ml by evaporation. The mycotoxin concentration in the extract was determined by TLC. When necessary, additional cleanup was performed for aflatoxin and ochratoxin A as described by Eppley (6). After addition of 1.5 ml of chloroform, 2 ml of the extract was applied to a kieselgel chromatographic column (1 by 13 cm) prepared by adding 2 g of sodium sulfate followed by 4 g of kieselgel 60 and topped off with another 2 g of sodium sulfate. The column was separately washed with acetone:benzene (5:95) and anhydrous ethylether and then eluted with 100 ml each of chloroform:methanol (97:3) and acetic acid:benzene (1:9) for aflatoxins and ochratoxin A, respectively. Separately, 2 ml of the chloroform extract was applied to a double layer of chromatographic column (1 by 20 cm) of 4 g of florisil (60 to 100 mesh; Sigma Chemical Co., St. Louis, Mo.) on top of 4 g of kieselgel. Sterigmatocystin was eluted from the column with 100 ml of benzene:methanol (9:1) after washing with 100 ml each of benzene:acetone (99:1) (18). The eluants were evaporated to dryness in an evaporator. The residues were dissolved in 0.5 ml of chloroform and applied to TLC plates for detection of each mycotoxin.

Adsorbosil-1 (0.25 thick; Applied Science Laboratories, State College, Pa.) was applied to each TLC plate and activated by heating at 80°C for 2 h. Ten to twenty microliters of each sample and the mycotoxin standard were spotted on a plate with a microsyringe. The plates were developed with chloroform:acetone (9:1) for aflatoxins and with benzene:acetic acid (9:1) for ochratoxin A (6), and benzene:methanol:acetic acid

(9:1:5) for sterigmatocystin (18). The fluorescence intensities of each mycotoxin spot were measured by fluorodensitometry (model MPF-2A; Hitachi Ltd., Tokyo, Japan).

Antimicrobial effect of OMCA. (i) Microorganisms. The antimicrobial spectrum of OMCA was tested with eight strains of bacteria, i.e., *Shigella sonnei* 75-17, *Salmonella typhimurium* 75-276, pathogenic *Escherichia coli* E-2602, *Proteus vulgaris* 75-3, *Pseudomonas aeruginosa* 75-27, *Clostridium botulinum* CB-24, *Clostridium perfringens* NCTC-8329, and *Staphylococcus aureus* 209P, and nine fungal strains, i.e., *Trichophyton mentagrophytes* stT-1, *Trichophyton rubrum* stT-2, *Epidermophyton floccosum* stT-3, *Microsporum gypseum* stT-5, *Microsporum canis* stT-6, *Aspergillus fumigatus* Kuboyama, *Aspergillus niger* stA-2, *Candida albicans* stT-1, and *Cryptococcus neoformans* stY-8 (stock culture in my laboratory).

(ii) Antibiotics. Chloramphenicol (Sankyo Co., Ltd., Tokyo, Japan) was used as the reference antibiotic. Chloramphenical was dissolved in sterilized distilled water at 1 mg/ml. OMCA was dissolved in sterilized dimethylsulfoxide in a sterile test tube at 2 mg/ml. Each solution was diluted in sequential two-fold dilutions with distilled water, and portions of diluted solutions were added to the agar plates or slants for drug sensitivity test at concentrations of 0.195 to 200 μg of antibiotics per ml.

(iii) Media. Heart infusion agar (Difco Laboratories, Detroit, Mich.) was used as drug sensitivity test plate for the determination of minimum inhibitory concentrations (MICs) against various bacteria, with the exception that 1-cysteine monohydrochloride at a 1% concentration was added for *Clostridium* species. Test bacteria were each preincubated in 10 ml of Tripto-soy broth (Eiken Chemical Co., Ltd., Tokyo, Japan) for 20 h at 37°C. A Sabouraud agar slant was used for the drug sensitivity test for fungi, with a Sabouraud agar slant or potato dextrose agar slant used for preincubation. Test fungi were each preincubated on the above medium for 7 days at 25°C.

(iv) MIC determination. MIC determinations were performed by the method standardized by the Japanese Association of Chemotherapy (12). The medium for sensitivity test (heart infusion agar) was dissolved in an Erlenmeyer flask and sterilized and maintained in a 50°C water bath. A 2-ml quantity of OMCA or chloramphenicol solutions of various concentrations prepared was added into 18 ml of this medium, mixed well, and then poured into a petri dish. After preparation of each plate, bacterial solutions prepared by Tripto-soy broth were diluted 10 times with sterilized physiological saline and streaked on the agar plate with a sterilized loop. The plates were incubated at 37°C for 20 h. Anaerobic incubation was carried out at 37°C in an Anaer Box (type AZ; Hirasawa Workes, Tokyo, Japan) for *Clostridium* species.

The MIC of OMCA against various fungi was determined as follows. One loopful of the test fungal spores was suspended in 10 ml of 0.1% Driwel-Sabouraud broth. One loopful of suspension was streaked on a Sabouraud agar slant containing OMCA at various concentrations with static incubation at 25°C. *Candida* growth was examined after 24, 72, and 120 h, and the growth of other fungi was evaluated after the incubation for 72 and 120 h, and MICs were calculated.

RESULTS

Inhibitory effect of chloroform extract against growth and toxin production by mycotoxin-producing fungi. Inhibitory effects of chloroform extract to aflatoxin-producing fungi are shown in Table 1. The chloroform extract completely inhibited growth of both *A. parasiticus* and *A. flavus* at 25.3 mg/10 ml of SL broth (equivalent to 1 g of powdered cinnamon). At a concentration of 2.53 mg or below, the extract showed no inhibitory effect on the growth and aflatoxin production of *A. parasiticus*. It inhibited the aflatoxin B_1 production by *A. flavus* at 0.253 mg/10 ml of SL broth, and completely inhibited the growth of *A. ochraceus* and *A. versicolor* at 25.3 mg/10 ml of YES broth.

Inhibitory effect of various fractions obtained during the purification process against growth and aflatoxin production by *A. flavus*. As shown in Table 2, the *n*-hexane soluble fraction derived from the chloroform extract showed a strong inhibitory effect. This fraction was then applied to a kieselgel 60 chromatographic column to yield three fractions, of which the benzene:chloroform eluate showed a strong inhibition. This fraction was then vacuum concentrated in an evaporator and redissolved in benzene. The solution was applied to an aluminum oxide column and eluted sequentially

TABLE 1. *Inhibitory effect of chloroform extract of cinnamon on growth and aflatoxin production by A. parasiticus and A. flavus*

Treatment (mg of extract per 10 ml of SL broth)	*A. parasiticus* NRRL-3000				*A. flavus* ATCC-28539			
	Mycelial wt		Aflatoxin B_1		Mycelial wt		Aflatoxin B_1	
	mg/10 ml	Inhibition (%)	μg/ml	Inhibition (%)	mg	Inhibition (%)	μg/ml	Inhibition (%)
25.3	0	100	0	100	0	100	0	100
2.53	277.5	0	5.2	0	259.4	7	2.0	87
0.253	288.3	0	5.2	0	278.1	0	2.5	83
0.0253	279.6	0	5.0	0	275.9	0	15.1	0
Control	275.1		4.9		279.5		15.0	

with benzene, benzene:chloroform (9:1), and chloroform. Inhibition was detected only in the chloroform eluate. This fraction was subjected to PTLC with a kieselgel G TLC plate, yielding six fractions. The inhibitory effect was detected only in the T-4 fraction. Gas chromatography-mass spectrometry analysis showed that the T-4 fraction contained three compounds as the result of comparison of the mass spectrum of the T-4 fraction with those of known compounds (47). One compound had the spectrum of CA, one compound had a spectrum similar to sesquiterpen alcohol, and one compound had a molecular weight of 162, with its spectrum different from any of the known compounds. The T-4 fraction was then subjected to another PTLC, yielding three fractions. Antifungal inhibition was detected in the TT-1 and TT-2 fractions, with the more pronounced effect found in TT-2 fraction.

TABLE 2. *Inhibitory effect of various fractions in the purification process of cinnamon on growth and aflatoxin production by A. flavus ATCC 28539*

Fraction	Mycelial wt		Aflatoxin B_1	
	mg/10 ml	Inhibition (%)	μg/ml	Inhibition (%)
Chloroform extract	0	100	0	100
n-Hexane soluble	0	100	0	100
n-Hexane insoluble	271.5	0	15.0	0
Silica gel column chromatography				
n-Hexane	281.3	0	15.5	0
Benzene:chloroform	0	100	0	100
Chloroform	264.3	2.2	14.8	1.3
Alumina column chromatography:				
Benzene	279.2	0	15.0	0
Benzene:chloroform	275.6	0	15.0	0
Chloroform	0	100	0	100
PTLC				
T-1	283.1	0	15.0	0
T-2	275.5	0	15.0	0
T-3	269.4	0	15.0	0
T-4	0	100	0	100
T-5	267.8	0	14.2	5.3
T-6	270.5	0	15.0	0
TT-1	164.0	40	2.0	87
TT-2	0	100	0	100
TT-3	274.2	0	15.0	0
Control	270.3		15.0	

Identification of the final products. The TT-2 fraction was analyzed by means of gas chromatography-mass spectrometry; *m/e* values obtained were 39, 51, 63, 65, 77, 91, 103, 119, 131, 147, and 162 (Fig. 2). The TT-2 fraction proved to be a single compound, and its molecular weight was estimated to be 162. The mass spectrum and TLC properties of TT-2 were compared with those of OMCA (molecular weight, 162), methyl cinnamate (molecular weight, 162), eugenol (molecular weight, 164), and other compounds derived from cinnamon and having a molecular weight around 162. The mass spectrum and TLC properties of the TT-2 fraction were identical with those of OMCA. The element analyses and melting point measurements gave identical values. This confirms the previous observation of Karig (10). The compound has the structure shown in Fig. 3, the molecular

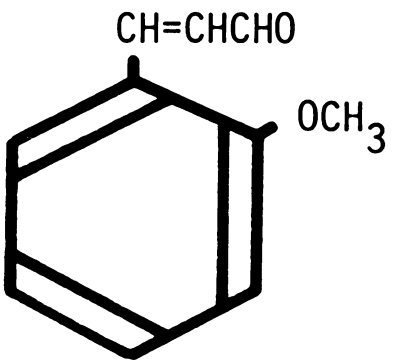

FIG. 3. *Structure of OMCA.*

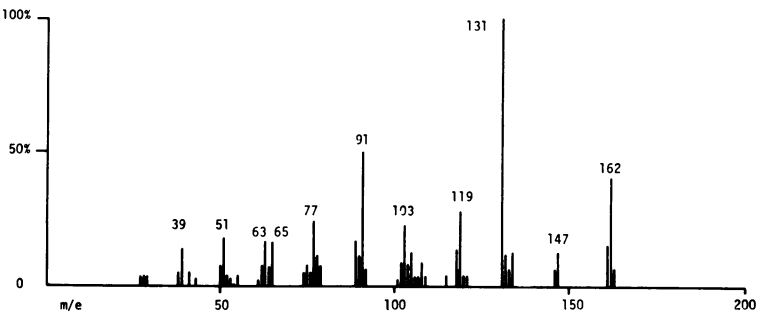

FIG. 2. *Mass spectrum of the TT-2 fraction.*

formula $C_{10}H_{10}O_2$, a molecular weight of 162, and mp 46 to 47°C. It forms pale yellow flakes soluble in various organic solvents but slightly soluble in water.

The mass spectrum of TT-1 fraction was compared with those of commercial CA, cinnamic acid, and cinnamic alcohol. They were identical, and thus the TT-1 fraction was identified as CA.

Inhibitory effect of OMCA against growth and toxin production by toxigenic fungi. As shown in Table 3, OMCA inhibited the growth of both *A. parasiticus* and *A. flavus* at 100 μg/ml and the growth of both *A. ochraceus* and *A. versicolor* at 200 μg/ml. At 6.25 μg/ml, OMCA inhibited the toxin production by two aflatoxin-producing species to about 90%. About 90% inhibition of the toxin production by ochratoxin A-producing and sterigmatocystin-producing fungi was observed at 25 μg/ml and 50 μg/ml, respectively. The MIC of the CA fraction for *A. flavus* was about four times that of OMCA, i.e., 400 μg/ml.

Antimicrobial effect of OMCA. Table 4 shows the MICs of OMCA against various bacteria, and Table 5 shows those against various fungi. OMCA completely inhibited the growth of *S. aureus* and *C. botulinum* at 200 and 100 μg/ml, respectively.

The MICs of OMCA for fungi are as follows: 3.12 μg/ml in *M. canis;* 6.25 μg/ml in *T. mentagrophytes, T. rubrum, E. floccosum,* and *M. gypseum;* 12.5 μg/ml in *C. neoformans;* 50 μg/ml in *C. albicans;* 100 μg/ml in *A. fumigatus;* and 200 μg/ml in *A. niger.* OMCA showed considerable inhibition against the five species of dermatophytoses in comparison with other fungi tested.

DISCUSSION

It has been reported that cinnamon oil contains CA, eugenol, cinnamic alcohol, cinnamic acid, linalool, and over 10 other compounds (10, 44–46). Of these compounds, CA, eugenol, cinnamic alcohol, and linalool have been thoroughly investigated by Okazaki et al. (35, 37–41) and Bullerman et al. (4). Okazaki et al. (39) indicated that nine different species of fungi, including pathogenic fungi, are inhibited by CA at concentrations from 7.8 to 500 μg/ml. Bullerman et al. (4) also reported that at concentrations greater than 200 and 150 μg/ml, CA inhibited completely the growth and the production of aflatoxins, respectively, by *A. parasiticus.*

During the course of the present study, two antimicrobial compounds were obtained as final compounds from the chloroform extract of cinnamon powder, CA, and OMCA. The latter was already reported by Karig (10) in Germany in

TABLE 3. *Inhibitory effect of OMCA on the growth and mycotoxin production by four strains of toxigenic fungi*

Treatment (μg of OMCA per ml)	*A. parasiticus* NRRL-3000				*A. flavus* ATCC-28539				*A. ochraceus* STA-15				*A. versicolor* I-20-b			
	Mycelial wt		Aflatoxin B_1		Mycelial wt		Aflatoxin B_1		Mycelial wt		Ochratoxin A		Mycelial wt		Sterigmatocystin	
	mg/10 ml	Inhibition (%)	μg/ml	Inhibition (%)	mg/10 ml	Inhibition (%)	μg/ml	Inhibition (%)	mg/10 ml	Inhibition (%)	μg/ml	Inhibition (%)	mg/10 ml	Inhibition (%)	μg/ml	Inhibition (%)
200	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
100	0	100	0	100	0	100	0	100	454.6	17	0.8	93	319.6	19	1.2	92
50	260.6	16	0.1	98	273.5	0	1.0	94	519.2	6	0.7	94	324.2	18	1.2	92
25	307.2	0	0.3	94	278.1	0	0.7	95	458.3	17	1.1	91	350.5	11	4.6	68
12.5	310.5	0	0.2	96	283.9	0	1.5	90	543.2	2	10.5	15	369.0	7	15.0	0
6.25	298.6	3	0.4	92	266.8	2	1.5	90	543.3	2	12.5	0	397.5	0	14.8	0
3.12	309.4	0	2.8	43	273.7	0	1.8	88	552.1	0	12.7	0	324.3	18	14.0	0
1.56	310.1	0	5.2	0	275.4	0	15.0	3	539.5	2	12.2	0	395.1	0	13.9	2
Control	309.1		5.0		273.3		15.5		549.4		12.3		394.9		14.2	

TABLE 4. *MIC of OMCA for various bacteria*

Organism	MIC of OMCA (μg/ml)	MIC of chloramphenicol (μg/ml)
S. sonnei	>200	0.39
S. typhimurium	>200	12.5
E. coli	>200	6.25
P. vulgaris	>200	12.5
P. aeruginosa	>200	200
S. aureus	200	3.12
C. perfringens	>200	3.12
C. botulinum	100	3.12

TABLE 5. *MIC of OMCA for various fungi*

Organism	MIC (μg/ml) of OMCA at:		
	1 Day	3 Days	5 Days
T. mentagrophytes		6.25	6.25
T. rubrum		6.25	6.25
E. floccosum		6.25	6.25
M. gypseum		6.25	6.25
M. canis		3.12	3.12
A. fumigatus		50	100
A. niger		200	200
C. albicans	25	50	50
C. neoformans		12.5	12.5

1975, but its antimicrobial effect was not investigated at that time. By using comparative antimicrobial data of CA (MIC, 400 μg/ml), OMCA has the highest inhibitory effect (MIC, 100 μg/ml) against growth of *A. flavus*. The OMCA inhibited the growth of three other strains of mycotoxin-producing fungi at concentrations from 100 to 200 μg/ml, and considerably inhibited the toxin production at concentrations from 6.23 to 50 μg/ml.

Moreover, it is very interesting that OMCA was effective only against *S. aureus* (MIC, 200 μg/ml) and *C. botulinum* (MIC, 100 μg/ml), but ineffective against other pathogenic bacteria (MIC, over 200 μg/ml), although it showed pronounced growth-inhibiting effect against pathogenic fungi. The MIC for five species of dermatophytoses was 3.12 to 6.25 μg/ml.

Providing its toxigenicity is low, utilization of this compound may be expected as an antifungal agent in foods and as a treatment of dermatomycosis.

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