Detection of Antifungal Activity in *Anemarrhena asphodeloides* by Sensitive BCT Method and Isolation of Its Active Compound

Yasuhiro Iida,[†] Ki-Bong Oh,[†] Mikako Saito,[†] Hideaki Matsuoka,*[,†] Hiroshi Kurata,[‡] Masahiro Natsume,[§] and Hiroshi Abe[§]

Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, Tokyo 184-8588, Japan, Center for Food and Environmental Sciences, The Tokyo Kembikyoin Foundation, 5-3-11, Maesawa, Higashi-kurume, Tokyo 203-0032, Japan, and Department of Applied and Biological Science, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

Antifungal activity was detected from *Anemarrhena asphodeloides* by the Bio-Cell Tracer (BCT) method. An active fraction was separated by silica gel column chromatography and reverse-phase HPLC. The molecular weight was determined by GC−MS, and the molecular structure was analyzed by IR, ^{1}H NMR, and ^{13}C NMR. The isolated compound was found to be identical to nyasol, (*Z*)-1,3-bis(4-hydroxyphenyl)-1,4-pentadiene, which formerly appeared in the literature without any remark on the antifungal activity. This compound showed antimicrobial activity against 38 strains of fungi and five strains of bacteria. The minimum inhibitory concentration (MIC) ranged from 12.5 to 200 μg mL^{-1}, except for two strains based on the broth dilution method.

Keywords: *Medicinal plant; Anemarrhena asphodeloides; antifungal activity; Bio-Cell Tracer; nyasol*

INTRODUCTION

There are intense needs for novel antifungal and antibacterial compounds applicable to food and feed preservatives. These compounds must have high activity against fungi and bacteria and low biotoxicity to human beings and animals. From this viewpoint, medicinal plants are promising resources. In scanning the literature, however, we found only a few reports describing the screening methodology of detection of antifungal activity in medicinal plants (Chand et al., 1994).

Previously, we reinvestigated medicinal plants by a sensitive single-cell assay method (Oh et al., 1996) and found that many plants contained active components. This unique assay system, a Bio-Cell Tracer (BCT), is now commercially available (Hidan Co., Ltd, Kashiwa, Japan). BCT can detect the growth rate change of a single hypha at every 30 s to a precision of 0.1 μm min^{-1} (Oh et al., 1993). Therefore, a transient decrease in the growth rate of a test hypha can be detected. The sensitivity of antifungal activity detection of this system was 100-fold higher than that of conventional methods (Oh et al., 1996).

Anemarrhena asphodeloides is a typical herbal medicine that antifungal activity was detected for the first time by this BCT method. Steroidal saponins (Kawasaki et al., 1963), xanthone C-glycosides (Takahashi et al., 1985), and polysaccharides were reported as chemical constituents of the rhizome of this title plant. And this rhizome is known to have anti-diabetic activity (Takahashi et al., 1985), diuretic activity (Saito et al., 1994), and molluscicidal activity (Takeda et al., 1989). And the boiled extract with several herbal medicines was widely used in Chinese and Japanese folk medicines. These suggest a low biotoxicity to humans when used at the proper concentration. Thus, we have tried to isolate the most active lead compound from this plant material. This paper describes the isolation procedure for the compound and its molecular characteristics.

EXPERIMENTAL PROCEDURES

Materials and General Procedure. Rhizomes of *A. asphodeloides* of medicinal grade were purchased from Tochimoto-Tenkaido Co., Osaka, Japan. Other chemicals and solvents were of analytical grade. Wakogel C-300 silica gel (Wako Pure Chemical Industries, Osaka) was used for the column chromatography. The F_{254} silica gel-coated glass plate (Merck, Darmstadt, Germany) was used for thin-layer chromatography (TLC). The coloring condition of the TLC plate was treated with a vanillin−sulfuric acid (VS) reagent at 100 °C for 3 min. ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and 2D NMR spectra were measured with A500 FT−NMR (JEOL Ltd., Tokyo) in a $CDCl_3$ solution. Chemical shifts were given in δ (ppm) values relative to tetramethylsilane (TMS) as an internal standard. Gas chromatography−mass spectrometry (GC−MS) spectra were measured with a Hewlett-Packard 5890 II gas chromatography linked to a JMS-SX102A mass spectrometer (JEOL Ltd.) (column: DB-1 column, J & W Scientific, 15 m × 0.25 mm; line: He flow at 1 mL min^{-1}; column temperature profile: 50 °C for 1 min, increasing from 50 to 250 °C at 10 °C min^{-1} and hold at 250 °C for 10 min; electron potential: 70 eV). Reverse-phase high-performance liquid chromatography (HPLC) was performed on a μ-Bondasphere column (5μ C_{18}-100 Å, 15 cm × 3.9 mm, Nihon Waters Ltd., Tokyo) in a Tosoh SC-8020 system (Tosoh Co., Tokyo). Detection was carried out with an UV detector at 254 nm (UV-8020, Tosoh Co., Tokyo). Optical rotation was taken on a DIP-1000 digital polarimeter (Jasco Co., Tokyo).

Isolation Procedure. The antifungal activity of each fraction obtained in the course of isolation was measured by the BCT method as described in Oh et al. (1996). To measure the antifungal activity of each fraction by BCT, the solvent of

* Tel: +81-42-388-7029, fax: +81-42-387-1503, e-mail: biofunc@cc.tuat.ac.jp.
[†] Department of Biotechnology.
[‡] Center for Food and Environmental Sciences.
[§] Department of Applied and Biological Science.

10.1021/jf980707t CCC: $18.00
Published on Web 01/05/1999

each fraction that was obtained in this procedure was removed by evaporation under reduced pressure. The residues were dissolved in dimethyl sulfoxide (DMSO) (20−40 mg mL^{-1}) as stock solutions. These stock solutions were diluted with potato dextrose broth (PDB) medium (Difco) to prepare the prescribed concentrations before use.

Pulverized rhizomes of *A. asphodeloides* (1000 g) were suspended in 60% aqueous acetone (2 L) for 3 days at room temperature. After insoluble residues were removed, the solvent was evaporated under reduced pressure to purge acetone. The extracted material was collected by lyophilization. The extracted material was dissolved in H_2O (500 mL) and extracted with two 500-mL portions of *n*-hexane (Hex), three 500-mL portions of ethyl acetate (EtOAc), and three 500-mL portions of 1-butanol (BuOH). The evaporation of each fraction resulted in the following amounts of dried matter 171.7 (H_2O), 1.59 (Hex), 1.82 (EtOAc), and 2.69 g (BuOH). Among these four fractions, the EtOAc fraction showed the highest antifungal activity; therefore, this fraction was further purified by the silica gel column chromatography (3.0 × 25 cm). When the column was eluted with $CHCl_3$−EtOAc (20:1), three distinct fractions (Fr-A, Fr-B, and Fr-C) were obtained. The weight of each fraction was 64.2 (Fr-A), 70.0 (Fr-B), and 774.0 mg (Fr-C), respectively. Among these three fractions, Fr-B showed the highest antifungal activity. When the Fr-B was developed on a TLC plate with MeOH−$CHCl_3$−H_2O (35:65:10) and colorized with VS reagent, only one spot appeared at R_f 0.7. Therefore, this fraction was applied on the reverse-phase HPLC and eluted with H_2O−CH_3CN with its ratio being varied from 68:32 to 29:71 during a 13-min elution at a flow rate of 1.0 mL min^{-1}. Finally 40.7 mg of dried matter was obtained as the purified matter.

Molecular Analysis Data of the Purified Matter. $[\alpha]_D$ − 134° (MeOH: c 0.47); IR ν_{max} 3350 cm^{-1}; EIMS: *m/z* = 252 (M^+, 100%), 237 (38), 158 (83), 145 (77), 131 (66), 107 (90), 77 (36). ^1H NMR ($CDCl_3$): δ 7.19 (2H, d, J = 8.5 Hz, H-2′,6′), 7.12 (2H, d, J = 8.5 Hz, H-2″,6″), 6.81 (2H, d, J = 8.5 Hz, H-3′,5′), 6.80 (2H, d, J = 8.5 Hz, H-3″,5″), 6.54 (1H, d, J = 11.0 Hz, H-1), 6.03 (1H, ddd, J = 6.0, 10.5, 16.5 Hz, H-4), 5.70 (1H, dd, J = 10.0, 11.0 Hz, H-2), 5.19 (1H, ddd, J = 1.5, 1.5, 16.5 Hz, H-5), 5.18 (1H, ddd, J = 1.5, 1.5, 10.5 Hz, H-5), 4.89 (2H, br s, exch. D_2O, -OH), 4.51 (1H, dd, J = 6.0, 10.0 Hz, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 154.4 (C-4′)[a], 153.9 (C-4″)[a], 140.7 (C-4), 135.7 (C-1″), 131.9 (C-2), 130,0 (C-2′,6′)[b], 129.9 (C-1′), 128.9 (C-2″,6″)[b], 128.6 (C-1), 115.4 (C-3″,5″)[c], 115.1 (C-3′,5′)[c], 115.0 (C-5), 46.8 (C-3). (The signals designated with the same letter may be interchanged.)

MIC Determination of the Purified Matter. To investigate the antifungal spectrum of the purified matter, its minimum inhibitory concentration (MIC) against various fungi was determined. The assay method was the microdilution method described below.

Test fungi were inoculated on a potato dextrose agar (PDA) medium (Difco, Detroit) and incubated at 28 °C for 1−2 weeks until they formed a well-extended fungal mat with spores. These spores were collected with 0.05% Tween 80 solution and washed with sterile distilled water. The spores were suspended in a Czapek dox broth medium (Difco) or a PDB medium (Difco). The spore concentration was adjusted at 10^6 spores mL^{-1}.

The purified matter was dissolved in DMSO (40 mg mL^{-1}) and stored at −10 °C. Before use, this stock solution was thawed and diluted with Czapek Dox broth medium to prepare test solutions at prescribed concentrations. Initially, the stock solution was 10-fold diluted, and then 2-fold dilution was repeated. A 90-μL aliquot of each test solution was placed in each well of a 96-well microplate (8 rows × 12 columns, one row per one strain of test fungus), and 10 μL of a spore suspension (10^6 spores mL^{-1}) of a test fungus was added to each well. The final concentration of the purified matter in those wells ranged from 400 to 6.3 μg mL^{-1}. Column 1 contained the highest concentration, and column 7 contained the lowest concentration. Columns 8 and 9 were used as controls; column 8 contained Czapek dox broth medium, spores, and DMSO, while column 9 contained Czapek dox broth medium alone. The microplate was incubated at 28 °C for 7 days. The lowest concentrations that inhibited the growth of each test fungus determined from each row were assigned as the MICs against the respective test fungi.

On the other hand, the purified matter was assayed also for antibacterial activity by the microdilution method. Test bacteria were incubated in a nutrient broth (Difco) at 37 °C for 12 h and then suspended in the same nutrient broth. The bacterial cell density was adjusted at 10^8 cells mL^{-1}. In the same way as fungi, the test solutions for antibacterial activity assay were prepared and dispensed in each well of a microplate. The bacterial cell density in each well was 10^7 cells mL^{-1} at the beginning. Other conditions were same as those for fungi. After the microplate was incubated at 37 °C for 2 days, the lowest concentrations that inhibited the growth of each test bacterium determined from each row were assigned as MICs against the respective test bacteria.

RESULTS AND DISCUSSION

Antifungal Activity of Each Fraction Obtained in the Course of Isolation. In the course of isolation, each fraction obtained at various steps was assayed for its antifungal activity against *Aspergillus niger*. As summarized in Figure 1A, a crude sample obtained by acetone extract (■) showed a marked activity. Then, this crude matter was further purified. The crude acetone extract was extracted with four solvents: water, *n*-hexane, EtOAc, or BuOH. These four fractions were assayed for antifungal activity. As shown in Figure 1A, the EtOAc fraction (▲) showed the most marked inhibiting effect, although the test hypha recovered its growth rate within 20 min. This EtOAc fraction was applied to a silica gel column, and consequently three fractions (Fr-A, Fr-B, and Fr-C) were obtained. Among them, Fr-B was found to be the most active and showed marked inhibitory effect against *A. niger* at 1.25 μg mL^{-1} (Figure 1B; ×). Hyphal growth rate was completely inhibited by the Fr-B concentration of 12.5 μg mL^{-1} (Figure 1B; ○). Fr-B was subjected to the reversed-phase HPLC to confirm the purity. The matter finally obtained maintained similar activity (Figure 1B; ■) to Fr-B.

Structural Elucidation. The purified matter was colorless oil. The molecular ion peak [M^+] appeared at *m/z* 252 in EIMS analysis. HR (high-resolution)-EIMS analysis of this peak showed that *m/z* was 252.1120. A feasible molecular formula was speculated as being $C_{17}H_{16}O_2$. The ^{13}C NMR spectrum in $CDCl_3$ solution displayed 13 carbon signals, four of which (δ 130.0, 128.9, 115.4, and 115.1) appeared to be of double intensity. ^1H NMR spectra confirmed the presence of eight aromatic ring protons present as the AA′BB′ system (δ 7,19 [2H], 7.12 [2H], 6.81 [2H], and 6.80 [2H]). These two observations suggested the presence of two para-disubstituted benzene groups in the structure of the compound. The presence of the 1,3-disubstituted 1,4-pentadiene system was assigned from the COSY spectrum (carbon−proton and proton−proton). The J value (11.0 Hz) of the 1,2-double bond in the 1,4-pentadiene system at δ 6.54 Hz (H-1) and δ 5.70 Hz (H-2) was indicative of a cis stereochemistry. An IR band at 3350 cm^{-1} and two protons (δ 4.89 Hz) exchangeable with two deuteriums of D_2O indicated the presence of hydroxyl groups. The chemical shift of these exchangeable protons suggested that these hydroxyl groups constituted the hydroxyphenyl groups. Therefore, the present compound was determined to be (*Z*)-1,3-bis(4-hydroxyphenyl)-1,4-pentadiene (Figure 2).

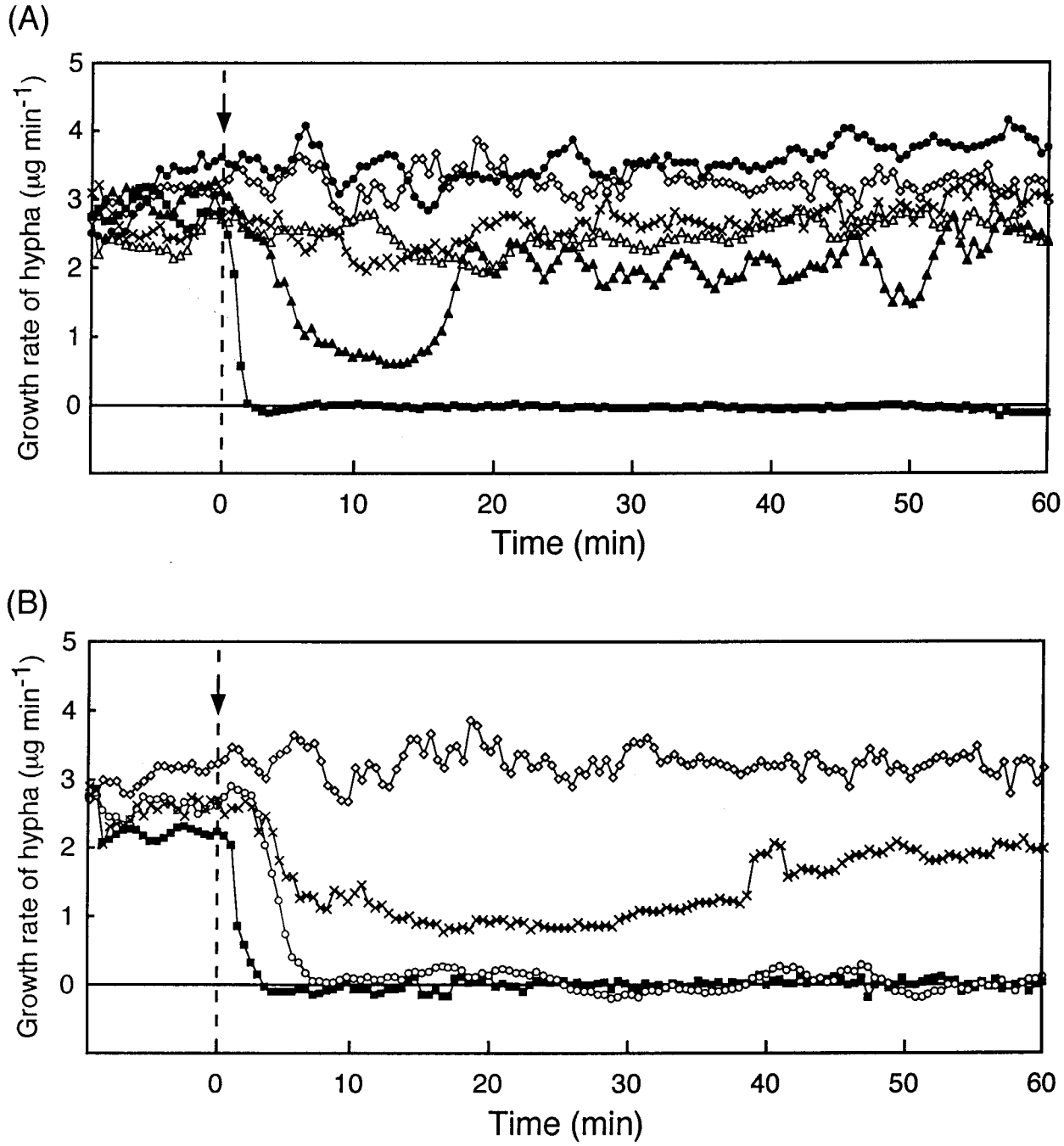

Figure 1. Antifungal activity of each fraction obtained at various steps of the isolation. The growth rate of a test hypha of *A. niger* was measured by the BCT method. At the arrow, PDB was replaced by a fresh PDB containing each fraction sample. (A) Fractions obtained by acetone extraction and successive extraction with various solutions: (◇) PDB (control), (■) crude acetone extraction fraction (500 μg mL^{-1}), (×) H_2O extraction fraction (12.5 μg mL^{-1}), (△) Hex extraction fraction (12.5 μg mL^{-1}), (▲) EtOAc extraction fraction (12.5 μg mL^{-1}), (●) BuOH extraction fraction (12.5 μg mL^{-1}). (B) Fraction obtained by a silica gel column chromatography and HPLC: (◇) PDB (control), (×) fraction B obtained by the elution with $CHCl_3$−EtOAc (20:1) in a Wakogel C-300 column chromatography (1.25 μg mL^{-1}), (○) 10-fold concentration of ×, (■) fraction obtained by a μ-Bondasphere column HPLC (12.5 μg mL^{-1}).

In scanning the literature, we found that nyasol has the same molecular structure. Nyasol has an asymmetric carbon atom at C-3. A specific rotation of (−)-nyasol isolated from *Hypoxis nyasica* (Marini-Bettolo et al., 1985) and (+)-nyasol isolated from *Asparagus cochinchinensis* (Tsui et al., 1996) was −147 and +112°, respectively. A specific rotation of the isolated compound in this study is −134°. From these results, an antifungal compound from *A. asphodeloides* is characterized as (−)-nyasol. Marini-Bettolo et al. also reported that nyasol was a chemical isomer of hinokiresinol isolated from *Chamaecyparis obtusa* (Hirose et al., 1965; Beracierta et al., 1976). The antifungal activity of the crude extract of *Araucaria angustifolia* containing 4.0−4.5% hinokiresinol was reported by Otsuka et al. (1995).

MIC of the Purified Matter against Fungi and Bacteria. MICs of nyasol against 38 strains of fungi and five strains of bacteria ranged from 12.5 to 400 μg mL^{-1}, as is shown in Table 1. *Curvularia lunata*, *Eurotium* sp., *Mucor* sp., and *Trychophyton* sp. showed lower MIC values (12.5−25.0 μg mL^{-1}) than other species. The MIC of cinnamic acid, which is used as a

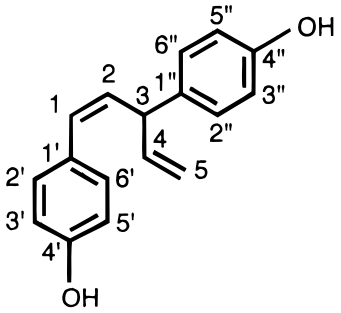

Figure 2. Structure of the purified compound. This structure was found to be equal to nyasol.

Table 1. Summary of MICs of the Purified Compound

strain of fungi[a]	MIC[c] (mg mL^{-1})
Aspergillus flavus HIC 6032	400
Aspergillus fumigatus HIC 6094	100
Aspergillus niger IFO 6661	200
Aspergillus oryzae 322-17	200
Aspergillus versicolor HIC 6247	200
Alternaria sp. HMC 1001	100
Candida albicans ATCC 10231	200
Candida tropicalis ATCC750	200
Cladosporium cladosporioides TSY 0030	50
Cladosporium sphaerospermum 728-1	50
Curvularia lunata M-1353-64	25
Fusarium solani HIC 5670	200
Fusarium oxysporium HIC 5651	50
Geotrichum candidum HIC 5700	200
Monilia sp. HIC 5745	200
Penicillium citrinum HIC 7043	200
Penicillium digitatum M-1393-16	200
Penicillium frequentans HIC 7104	200
Penicillium islandicum	200
Paecilomyces variotii 581	200
Scopulariopsis brevicaulis 629-3	100
Trichoderma sp. 101	50
Trichoderma viride M-5081-6	50
Trichophyton mentagrophytes HIC 8185	12.5
Trichophyton mentagrophytes 40996	12.5
Trichophyton mentagrophytes QM 248	25
Trichophyton rubrum NIH J	25
Trichophyton rubrum IFO 9185	12.5
Trichophyton rubrum IFO 6204	25
Saccharomyces cerevisiae IFO 1234	200
Byssochlamys nivea IFO 31351	100
Chaetomium globosum IFO 6347	200
Emericella nidulans C-28-2	100
Eurotium sp. HIC 6343	25
Eurotium rubrum M-401	100
Neurospora crassa HIC 5674	50
Mucor sp. 101	25
Rhizopus stlonifer IFO 30816	200

strain of bacteria[b]	MIC[d] (μg mL^{-1})
Bacillus subtilis IFO 13722	100
Escherichia coli ATCC 8739	>400
Proteus vulgaris ATCC 6380	25
Pseudomonas aureginosa	100
Staphylococcus aureus ATCC 65389	50

[a] Potato dextrose broth was used for the growth of *Candida* sp., *Trychophyton* sp., *S. cerevisiae*, and *R. stolonifer*. Czapek dox medium was used for the other strains. [b] Nutrient broth was used for the growth of bacteria. [c] MICs against fungi were decided after 7 days incubation. [d] MICs against bacteria were decided after 2 days incubation.

food additive in Japan, was 2 mg mL^{-1} against *Staphylococcus aureus* and *Escherichia coli* and was 500 μg mL^{-1} against *Saccharomyces cerevisiae* and *Aspergillus oryzae* (Ueda et al., 1982), and one of its derivative (the cis dimer of octanol−cinnamic acid esters) was 156 μg mL^{-1} against *Candida albicans* (Ulubelen et al., 1996). The MIC of catechin was higher than 200 μg mL^{-1} against *Pseudomonas aeruginosa* (Kayser et al., 1997). The MIC of vanillin was 1250 μg mL^{-1} against *C. albicans* (Boonchird et al., 1982). In comparison with these data, the antimicrobial activity of nyasol is not inferior and its antimicrobial spectrum was wide. Moreover, the component is expected to be less toxic to humans, taking into account their frequent use in traditional medicine.

LITERATURE CITED

Beracierta, A. P.; Whiting, D. A. Synthesis of the Dimethyl ethers of Agatharesinol, Sequirin-A, and Hinokiresinol, Related Norlignans of Coniferae. *Tetrahedron Lett.* **1976**, *27*, 2367−2370.

Boonchird, C.; Flegel, T. W. *In vitro* Antifungal Activity of Eugenol and Vanillin against *Candida albicans* and *Cryptococcus neoformans*. *Can. J. Microbiol.* **1982**, *28*, 1235−1241.

Chand, S.; Lusunzi, I.; Veal, D. A.; Williams, L. R.; Karuso, P. Rapid Screening of the Antimicrobial Activity of Extracts and Natural Products. *J. Antibiot.* **1994**, *47*, 1295−1304.

Hirose, Y.; Oishi, N.; Nagaki, H.; Nakatsuka, T. The Structure of Hinokiresinol. *Tetrahedron Lett.* **1965**, *41*, 3665−3668.

Kawasaki, T.; Yamauchi, T.; Saponins of Timo. *Chem. Pharm. Bull.* **1963**, *11*, 1221−1224.

Kayser, O.; Kolodziej, H. Antibacterial Activity of Extracts and Constituents of *Pelagonium sidoides* and *Pelargonium reniforme*. *Planta Med.* **1997**, *63*, 508−510.

Marini-Bettolo, C. B.; Nicoletti, M.; Messana, I. Research on African Medicinal Plants-X. *Tetrahedron* **1985**, *41*, 665−670.

Oh, K.-B.; Matsuoka, H.; Nemoto, Y.; Sumita, O.; Takatori, K.; Kurata, H. Determination of anti-*Aspergillus* Activity of Antifungal Agents Based on the Dynamic Growth Rate of a Single Hypha. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 363−367.

Oh, K.-B.; Iida, Y.; Matsuoka, H.; Kurata, H. Rapid and Sensitive Screening of Antifungal Activity in Medicinal Plants by a Single-cell Biosensing System. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 911−913.

Otsuka, K.; Sakurada, K.; Nakamura, E. Japanese Patent JP 07025719 A2, 1995

Saito, S.; Nagase, S.; Ichinose, K. New Steroidal Saponins from the Rhizomes of *Anemarrhena asphodeloides* BUNGE (Liliaceae). *Chem. Pharm. Bull.* **1994**, *42*, 2342−2345.

Takahashi, M.; Konno, C.; Hikino, H. Isolation and Hypoglycemic Activity of Anemarans A, B, C, and D, Glycans of *Anemarrhena asphodeloides* Rhizomes. *Planta Med.* **1985**, *51*, 100−102.

Takeda, O.; Tanaka, S.; Yamasaki, K.; Kohda, H.; Iwanaga, Y.; Tsuji, M. Screening for Molluscicidal Activity in Crude Drugs. *Chem. Pharm. Bull.* **1989**, *37*, 1090−1091.

Tsui, W.-Y.; Brown, G. D. (+)-Nyasol from *Asparagus cochinchinensis*. *Phytochemistry* **1996**, *43*, 1413−1415.

Ueda, S.; Yamashita, H.; Nakajima, M.; Kuwabara, Y. Inhibition of Microorganisms by Spice Extracts and Flavouring Compounds. *Nippon Shokuhin Kogyo Gakkaishi* **1982**, *29*, 111−116 (in Japanese).

Ulubelen, A.; Sonmez, U.; Topcu, G.; Bozok, J., C. An abietane diterpene and two phenolics from *Salvia forskahlei*. *Phytochemistry* **1996**, *42*, 145−147.

Received for review June 30, 1998. Revised manuscript received November 2, 1998. Accepted November 6, 1998.

JF980707T