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Liquid Chromatography-Mass Spectrometry-Based Chemotaxonomic Classification of *Aspergillus* spp. and Evaluation of the Biological Activity of Its Unique Metabolite, Neosartorin

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This work aimed to classify *Aspergillus* (8 species, 28 strains) by using a secondary metabolite profile-based chemotaxonomic classification technique. Secondary metabolites were analyzed by liquid chromatography ion-trap mass spectrometry (LC-IT-MS) and multivariate statistical analysis. Most strains were generally well separated from each section. *A. lentulus* was discriminated from the other seven species (*A. fumigatus, A. fennelliae, A. niger, A. kawachii, A. flavus, A. oryzae,* and *A. sojae*) with partial least-squares discriminate analysis (PLS-DA) with five discriminate metabolites, including 4,6-dihydroxymellein, fumigatin, 5,8-dihydroxy-9-octadecenoic acid, cyclopiazonic acid, and neosartorin. Among them, neosartorin was identified as an *A. lentulus*-specific compound that showed anticancer activity, as well as antibacterial effects on *Staphylococcus epidermidis*. This study showed that metabolite-based chemotaxonomic classification is an effective tool for the classification of *Aspergillus* spp. with species-specific activity.

Keywords: *Aspergillus lentulus*, liquid chromatography ion-trap mass spectrometry (LC-IT-MS), secondary metabolite, neosartorin, antibacterial activity, anticancer activity

Introduction

Aspergillus is one of most important fungi thriving in soil, plant debris, outdoor and indoor air, water, wood, and decaying materials [30]. *Aspergillus* species such as *A. flavus*, *A. ochraceus*, *A. fumigatus*, *A. niger*, *A. sojae*, and *A. parasiticus* are pathogenic to most animal species, including humans, often causing food spoilage and concomitantly producing bioactive and/or toxic secondary metabolites [12, 38]. Mycotoxins, the toxic metabolites produced by *Aspergillus* spp., possess pharmacological activities such as antibiotic effect, growth promoters, and drugs [4, 37]. *A. flavus* and *A. fumigatus* are known to produce aflatoxins and gliotoxin [21, 34], whereas *A. oryzae* and *A. sojae* have been widely used in the fermentation of various food products [26].

Fungal identification has been generally based on the morphological, physiological, and chemical characteristics of specimens [1]. Although more than 250 species of *Aspergillus* have been studied in the past several centuries [13], the use of taxonomy alone may not be sufficient in fully classifying *Aspergillus* strains. Recently, several studies, including our earlier studies, using a polyphasic approach for chemotaxonomy have been reported. Hong *et al.* [15] showed the taxonomical position of *A. fumigatus* and its related species by using macro- and micro-morphology, growth temperature regimes, extrolite patterns, and DNA sequence analysis. Kang *et al*. reported a chemotaxonomic classification of *Trichoderma* species using mass spectrometry (MS)-based metabolite profiling together with internal transcribed spacer (ITS) sequence analysis [18], and classified *Aspergillus* species according to culture medium-dependent and incubation time-dependent secondary metabolite profiling by LC-MS [17]. Among these polyphasic methods for fungal classification, the MS-based method has been widely used in studying the physical, chemical, and biological properties, and in analyzing the quantitative and qualitative features of various compounds. Liquid chromatography-

mass spectrometry (LC-MS), gas chromatography (GC)-MS, and capillary electrophoresis (CE)-MS are frequently used MS-based techniques [8]. Specifically, LC-MS is an effective technique for analyzing fungal secondary metabolites. Rank *et al.* [32] recently reported the results of a comparative analysis of secondary metabolite production between *A. oryzae* and *A. flavus* using LC-MS. Together with these metabolite profile analyses, many researchers have extensively studied the relationship between metabolites and bioactivities [19].

Comparative analyses of primary and secondary metabolites from a few *Aspergillus* species have earlier been performed using MS-based techniques [5, 28]. These metabolites are known to possess antioxidant, antifungal, antimicrobial, antiviral, and antitumor activities [2, 9, 42]. Although several studies on *Aspergillus* have been carried out in the past several decades, correlations between metabolite and activity remain elusive. This study focused on the application of a chemotaxonomic approach in classifying eight *Aspergillus* species (28 strains), including *A. fennelliae*, *A. flavus*, *A. fumigatus*, *A. kawachii*, *A. lentulus*, *A. niger*, *A. oryzae*, and *A. sojae*, based on metabolite profiling using the LC-IT-MS technique. We also evaluated the antimicrobial and anticancer activities of metabolites derived from *A. lentulus*.

Materials and Methods

Chemicals and Reagents

HPLC-grade water, methanol, and acetonitrile were purchased from Fisher Scientific (USA). Formic acid and brain heart infusion broth (BHB) were obtained from Sigma Aldrich (USA). Malt extract agar (MEA) was purchased from Becton Dickinson (USA). For cell culture, RPMI-1640 culture media and fetal bovine serum (FBS) were purchased from Gibco-BRL (USA). Penicillin-streptomycin (PS) was purchased from Invitrogen (USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. (USA).

Fungal Strains and Culture Conditions

A total of 28 *Aspergillus* strains included in this study are listed in Table 1. The strains were obtained from the Korean Agricultural Culture Collection (KACC, Republic of Korea), Korean Culture Center of Microorganisms (KCCM, Republic of Korea), and Korean Collection for Type Cultures (KCTC, Republic of Korea). The fungal strains were cultured on MEA plates for 3 days at 28°C in the dark. From the plate, a young mycelium piece (6 mm diameter) was transferred onto a fresh MEA plate and incubated for 12 days at 28°C in the dark. *Staphylococcus epidermidis* was cultured in a brain heart agar (BHA) plate for 7 days at 36°C.

Fungal Extraction

Upon optimal growth of the fungal strains on the agar medium, a total of 65 agar plates were harvested using ethyl acetate (50 ml per plate) and vortexed at 200 rpm for 9 h at 28°C. The extract solution was then evaporated using a speed-vacuum machine.

Table 1. Classification of *Aspergillus* according to its antibiotic activity against *Staphylococcus epidermidis.*

No.	*Aspergillus* species	Strain[a]	Section	Microorganisms[b]
				S. epidermidis
1	*A. fumigatus*	KACC 41186	*Fumigati*	-
2	*A. fumigatus*	KACC 41190		+
3	*A. fumigatus*	KACC 41191		-
4	*A. fumigatus*	KACC 41193		-
5	*A. fumigatus*	KACC 41196		+
6	*A. fumigatus*	KACC 41200		-
7	*A. fennelliae*	KACC 41125		-
8	*A. fennelliae*	KACC 41126		-
9	*A. lentulus*	KACC 41939		+
10	*A. lentulus*	KACC 41642		+
11	*A. lentulus*	KACC 41681		+
12	*A. niger*	KCCM 60318	*Nigri*	-
13	*A. niger*	KACC 40280		-
14	*A. niger*	KCTC 6960		-
15	*A. niger*	KCCM 32005		+
16	*A. kawachii*	KCCM 11459		-
17	*A. kawachii*	KCCM 32819		-
18	*A. flavus*	KCTC 16682	*Flavi*	-
19	*A. flavus*	KACC 40232		-
20	*A. flavus*	KCTC 6984		-
21	*A. oryzae*	KCCM 11896		-
22	*A. oryzae*	KACC 40067		-
23	*A. oryzae*	KACC 40242		-
24	*A. oryzae*	KACC 40234		-
25	*A. sojae*	KCCM 60354		-
26	*A. sojae*	KCTC 6376		-
27	*A. sojae*	KACC 41867		-
28	*A. sojae*	KACC 40072		-

[a]KACC, Korean Agricultural Culture Collection.
KCCM, Korean Culture Center of Microorganisms.
KCTC, Korean Collection for Type Culture.
[b]Antibacterial activity: + indicates antibacterial activity; - indicates no activity

LC-IT-MS/MS Analysis of *Aspergillus* Metabolites

Liquid chromatographic analysis was performed using a Varian 500-MS ion trap mass spectrometer (Varian, USA), which consisted of a LC pump (Varian 212), an auto sampler (Prostar 410), and a photodiode array detector (Prostar 335). The LC system was equipped with a Varian PurSuit XRs C18 column (100 × 2.0 mm

i.d., 3 µm). Mobile phases consisted of water (A) and acetonitrile (B) with 0.1% formic acid (v/v). The column was first eluted with 10% B for 2 min, and then gradient-eluted to 100% B for 28 min. The mobile phase was maintained at 100% B for 5 min, sharply returned to 10% B for 0.06 min, and then maintained for 5 min. Ten microliters of test samples was injected and the flow rate was set to 0.2 ml/min. The full-scan mass spectral range was 100–1,000 *m/z*. The operating parameters for analyzing the sample were as follows: spray needle voltage, 5 kV; capillary voltage, 70 V; drying temperature, 350°C; drying gas (nitrogen) pressure, 10 psi; nebulizer gas (air) pressure, 35 psi. Tandem mass spectrometry analysis was carried out using scan-type turbo data-dependent scanning (DDS) under the same conditions.

Ultra-Performance Liquid Chromatography-Quadruple Time-of-Flight Mass Spectrometry (UPLC-Q-TOF-MS) Analysis

UPLC-Q-TOF-MS was performed using the Waters Micromass Q-TOF Premier with UPLC Acquity System (Waters, USA) equipped with an Acquity UPLC BEH C18 column (100 × 2.1 mm., i.d., 1.7 µm; Waters, USA). The mobile phase was a modified version of that operated in the LC-IT-MS/MS system. The samples were separated using a linear gradient consisting of water (A) and acetonitrile (B) with 0.1% (v/v) formic acid under the following conditions: 0% B for 0.3 min, gradually increased to 30% B for 3 min, increased to 40% B for 1 min, and then increased to 100% B for 8 min. The sample injection volume was 5 µl and the flow rate was maintained at 0.3 ml/min. The full-scan mass spectral range was 100–1,000 *m/z*. The running parameters were as follows: capillary voltage, 28 kV; ion source temperature, 200°C; desolation gas flow, 600 L/h; cone gas flow, 50 L/h; and cone voltage, 35 V.

Data Processing and Multivariate Statistical Analysis

LC-IT-MS data were analyzed using the MS workstation software (version 6.9; Varian, USA). The raw (*.xms) files were converted into the network common data form (netCDF, *.cdf) formats using Vx Capture software (version 2.1; Adron, USA). After conversion, the netCDF files were aligned with the mass spectrometry by the MetAlign software package (http://www.metalign.nl) [23]. The resulting aligned *.cdv file was then transferred to a Microsoft Excel data sheet for sequential multivariate analysis.

Multivariate statistical analysis was performed using the SIMCA-P+ 12.0 (Umetrics, Sweden) and R+ packages (R Foundation for statistical Computing, Austria). The log-transformed MS data were mean-centered with unit variance scaling for statistical analysis. Principal component analysis (PCA) and partial least-squares discriminate analysis (PLS-DA) were employed to determine the distribution of *Aspergillus* strains. The strains were assigned to groups according to the results of homologous metabolite profiling that coincided with the hierarchical clustering analysis (HCA). PLS-DA separated the groups from the HCA and was a variable that was determined on the basis of the variable importance of the projection (VIP > 1.0) value and *p*-value ($p < 0.05$). The *p*-value between different metabolites-based cluster groups was determined using Statistica 7 (StatSoft, Inc., USA).

Isolation and Identification of Active Compound with Antimicrobial Activity

The preparative high-performance liquid chromatography (prep-HPLC) equipped with a C18 reversed-phase column (YMC-Pack Pro C18, 250 × 4.6 mm) was used to finally purify the active compound from the ethyl acetate extract of *A. lentulus* KACC 41681. The gradient elution of the mobile phase consisted of 5% acetonitrile in water (A) and 100% acetonitrile (B) and was programmed as follows: 0–60 min, gradually increased from 5% B to 100% B; 60–70 min, 100% B; and 70–80 min, sharply reduced to 5% B. The structure of the active compound was determined based on the reference and spectral data obtained using nuclear magnetic resonance (NMR), LC-ESI-MS-MS, and UPLC-Q-TOF-MS [29].

Compound: Yellow powder, M.W. 680.63, MS *m/z*: 680[M]$^+$, 621[M-59], 561[M-59-60], 501[M-59-60-60], 455, 281, 169; UV: λ_{max}/MeOH: 265 (674), 279 (762), 286 (719), 336; ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 13.85 (C-8-OH, s), 13.71 (C-1′-OH, s), 11.49 (C-1-OH, s), 11.30 (C-8′-OH, s), 7.10 (1H, d, *J* = 8.46 Hz), 6.52 (1H, d, *J* = 8.46 Hz), 6.41 (1H, s), 5.21 (1H, d, *J* = 1.26 Hz), 4.29 (1H, t, *J* = 1.38, 1.86 Hz), 3.72 (3H, s), 3.59 (3H, s), 2.84 (1H, m), 2.69 (C-5-OH, s), 2.37 (3H, m), 2.30 (H, m), 2.16 (H, m), 2.08 (C-3-CH3, s), 1.98 (H, m), 1.92 (C-5′-OCOCH3, s), 0.86(3H, d, *J* = 6.3 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ_c: 17.3 (C-3′), 20.5 (C-5′-OCOCH3), 21.3 (C-3-CH3), 23.3 (C-6), 24.6 (C-7), 28.0 (C-6′), 33.0 (C-7′), 53.5 (C-12′), 53.8 (C-12), 67.3 (C-5), 69.7 (C-5′), 82.3 (C-10′), 84.1 (C-10), 100.4 (C-9′), 100.7 (C-9), 105.0 (C-9b), 107.1 (C-9′b), 109.2 (C-4), 110.5 (C-2′), 114.9 (C-4′), 118.8 (C-2), 140.2 (C-3′), 148.7 (C-3), 155.7 (C-4′a), 157.1 (C-4a), 160.1 (C-1), 162.0 (C-1′), 169.3 (C-5′-OCO), 170.8 (C-11′), 171.4 (C-11), 177.9 (C-8′), 178.9 (C-8), 187.7 (C-9a), 188.0 (C-9a′). The active compound was identified as neosartorin ($C_{34}H_{32}O_{15}$) through spectral analysis and is shown in Fig. 3.

Paper Disc Method and Minimum Inhibitory Concentration Test for Determination of Antibacterial Activity

The agar diffusion method was employed to measure the antibacterial activity of the *Aspergillus* extract based on the resulting clear zone diameter produced in the presence of *S. epidermidis.* The inoculums (100 µl) were sprinkled and uniformly diffused on the BHA medium. The paper discs (6 mm in diameter) were covered with *Aspergillus* extract and its fractions dissolved in 100% methanol. The paper discs were soaked with the test sample and placed on the surface of the agar media and the plates were incubated at 37°C. After incubation, the antibacterial activity of the samples was determined by measuring the clear zone diameter surrounding each paper disc.

To accurately measure the antibacterial activity of the active compound in an *Aspergillus* extract, the minimum inhibitory concentration (MIC) test was performed. MICs were determined

by using a modified version of the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [11, 14]. Briefly, *S. epidermidis* was cultured in the BHB medium and collected. The inoculums were diluted to 1:20 (v/v), and then each well was seeded with 200 µl of diluted inoculums in a 96-well plate. Each well was treated with the test sample and serially diluted 2-fold to obtain a final sample concentration range from 3.9 to 1,000 µg/ml. The plates were incubated for 24 h at 37°C and the absorbance was measured at a wavelength of 570 nm using a microplate reader. The MIC was defined as the lowest concentration that results in a 90% reduction in bacterial growth [25]. Kanamycin was used as the positive control.

Cell Cultures and Conditions

A549 human lung carcinoma was purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium supplemented with 1% PS and 10% FBS and maintained at 37°C and 5% CO_2 atmosphere. During incubation, the culture medium was changed every 2 days.

Proliferation of A549 Cells Treated with Neosartorin

A549 lung carcinoma cells were seeded into a 96-well plate at a concentration of 2×10^4 cells/well. Twenty-four hours later, each well was renewed with fresh medium and treated with test samples at concentrations of 10, 50, and 150 µM (final concentration) for 24 h. Cell viability was assessed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Following the 24 h incubation with the appropriate test samples, MTT (0.1 mg/ml in PBS) was added to each well, incubated for 2 h at 37°C, and the substrate-containing medium was removed at the end of the incubation. Approximately 100 µl of DMSO was added to each well to dissolve the formazan crystals. The plates were shaken for 30 min at room temperature, and the absorbance was measured at a wavelength of 570 nm using a microplate reader. Cell viability was calculated and expressed as a percentage of the control.

Statistical Analysis

All data were expressed as the mean ± SE. To compare individual treatments to the controls, the Student's *t*-test was used. Statistical significance was set at $p < 0.05$.

Results

Metabolite-Based Chemotaxonomy of *Aspergillus*

Aspergillus extracts were analyzed by LC-IT-MS, and then the PCA score plot and dendrogram were constructed based on the data collected from the metabolites. In the PCA model, both the species and the section were clustered (Fig. 1A). The PC1 and PC2 scores explained 13.5% and 12.0% of the total variation observed in the analysis. The *Flavi*, *Fumigati,* and *Nigri* sections were separated by PC1, except for *A. kawachii*. As shown in Fig. 1B, the dendrogram

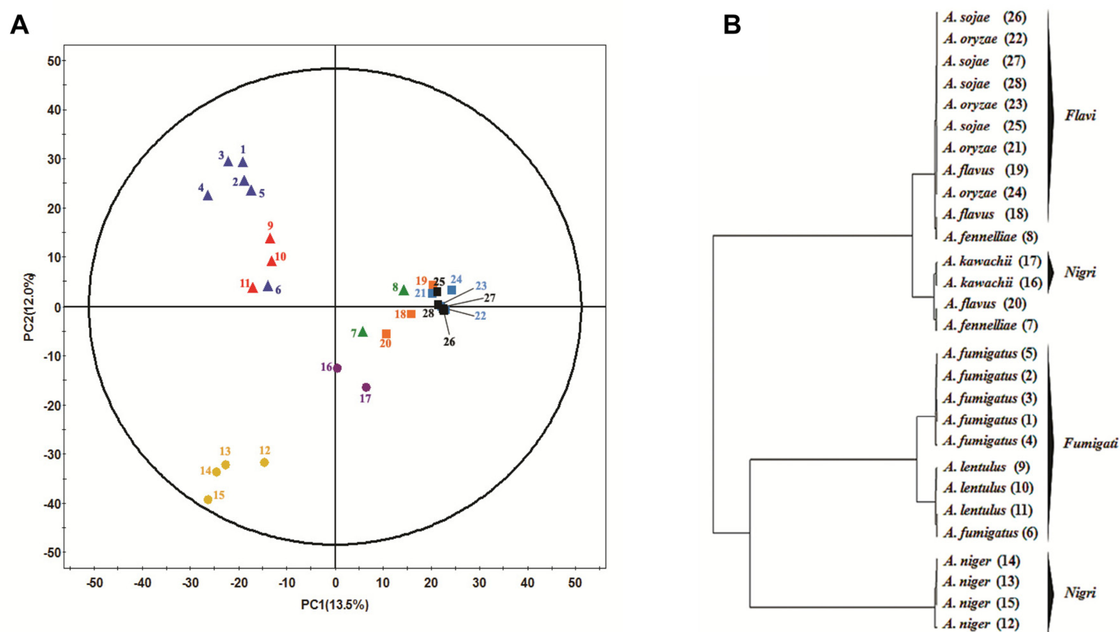

Fig. 1. Principal component analysis (PCA) score plot (**A**) and hierarchical clustering analysis (HCA) dendrogram (**B**) derived from the LC-IT-MS dataset of *Aspergillus* spp.

Fumigati section: ▲ *A. fumigatus*, ▲ *A. fennelliae*, ▲ *A. lentulus*; *Nigri* section: ● *A. niger*, ● *A. kawachii*; *Flavi* section: ■ *A. flavus*, ■ *A. oryzae*, ■ *A. sojae*. The numbers are listed in Table 1.

was divided mainly into two characteristic branches. The first branch included section *Flavi* (*A. oryzae*, *A. flavus*, and *A. sojae*) and *A. kawachii* of section *Nigri*, and these could be further subdivided. The second branch consisted of section *Fumigati* (*A. lentulus* and *A. fumigatus*) and *A. niger* of *Nigri* section, and this was again further subdivided. Although most strains were generally well separated from each section, *A. fennelliae* and *A. flavus* were particularly mixed with the other sections.

Antibacterial Activity of *Aspergillus*

Ethyl acetate extracts from 28 strains of *Aspergillus* were examined using the agar diffusion test, which measured antibacterial activity through the production of a clear zone against *S. epidermidis.* The results showed that two strains (KACC 41190, 41196) of *A. fumigatus,* three strains (KACC 41939, 41642, 41681) of *A. lentulus*, and one strain (KCCM 32005) of *A. niger* showed antibacterial activity against *S. epidermidis* (Table 1). The three *A. lentulus* strains showed an inhibitory effect on microbial growth. To isolate and elucidate the antibacterial components of the *A. lentulus* extract, one strain (KACC 41681), which showed the highest antibacterial activity, was selected and subjected to HPLC. Through comparison of all spectroscopic data with published values, the active compound against *S. epidermidis* was identified as neosartorin.

Identification of Significantly Discriminable Secondary Metabolites Between *A. lentulus* and the Other seven Species

Among the 28 *Aspergillus* strains analyzed, all *A. lentulus* strains showed antibacterial activity against *S. epidermidis*. Therefore, the PLS-DA model ($R^2X = 0.215$, $R^2Y = 0.986$, $Q2 = 0.878$) was used to determine the variables that differentiate *A. lentulus* from the other seven species. The potential variables were selected according to VIP (VIP > 1.0) and *p* values ($p < 0.05$), resulting in the identification of 11 significantly different metabolites. Metabolites were tentatively identified using UV λ_{max}, MS^n, UPLC-Q-TOF-MS analysis and references. Five of these metabolites were tentatively identified as 4,6-dihydroxymellein, fumigatin, 5,8-dihydroxy-9-octadecadienoic acid, cyclopiazonic acid, and neosartorin, whereas six peaks remained unidentifiable**.** The structures of the tentatively identified metabolites are shown in Fig. 3. In addition, the production of 11 significantly different metabolites was confirmed by calculating the peak intensities from an LC-IT-MS chromatogram (Table 3). Most of the significantly different metabolites were produced from section *Fumigati*. Specifically, *A. lentulus* produced nine metabolites except for fumigatin and one unidentified compound. However, fumigatin was only detected in *A. fumigatus*.

Antibacterial and Antiproliferative Effects of Neosartorin

The antibacterial activity of neosartorin against *S. epidermidis* is presented in Fig. 4. The MIC of neosartorin against *S. epidermidis* was 125 μg/ml, with an inhibitory effect on microbial growth at a higher concentration than that of the positive control, kanamycin (100 μg/ml). The antiproliferative effect of neosartorin on A549 cells, as evaluated by using the MTT assay, is shown in Fig. 5. Neosartorin significantly suppressed cell proliferation in A549 cells in a dose-dependent manner and was more effective than gallic acid under this experimental condition.

Discussion

In this study, *Aspergillus* species were chemotaxonomically classified into sections or species based on the secondary metabolite profiles using LC-IT-MS. In addition, several *Aspergillus* strains showed inhibitory activity against *S. epidermidis* (Table 1). The antimicrobial activities of *Aspergillus* spp. against various fungal species, including *Candida albicans*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Enterococcus faecalis* have been previously reported [36]. This study, to our knowledge, serves as the first report that describes the antibacterial effect of *Aspergillus* against *S. epidermidis*. All the tested strains of *A. lentulus* showed antibacterial activities against *S. epidermidis*. To distinguish *A. lentulus* from the other seven species on the basis of species-specific metabolites, the PLS-DA model was used (Fig. 2) and five significantly different metabolites were tentatively identified (Table 2). Several metabolites from *Aspergillus* have been previously reported. 4,6-Dihydroxymellein was first isolated from *Phomopsis helianthi* [3] and recently reported in *Aspergillus terreus* grown in a high-salt medium [41]. Although a large amount of this metabolite was detected in all strains of *A. lentulus*, this study showed that 4,6-dihydroxymellein did not impart any antibacterial effects on *S. epidermidis*.

Fumigatin, a quinone pigment produced by *A. fumigatus*, is well known for its antibacterial properties [40]. Fumigatin was detected in all strains of *A. fumigatus*; specifically, KACC 41186 produced the most amount of fumigatin compared with the other strains. Although *A. fumigatus* produced antibiotic components such as fumigatin, only two *A. fumigatus* strains showed antibacterial activity against *S. epidermidis* (Table 1). Our results suggest that the antibacterial

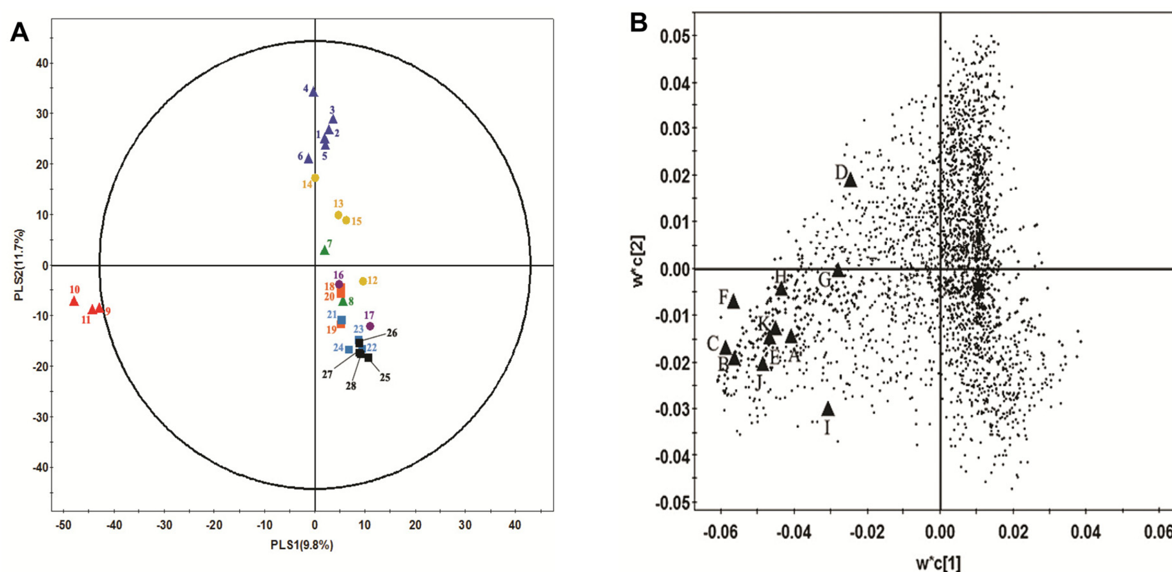

Fig. 2. Partial least-squares discriminate analysis (PLS-DA) score plot (**A**) and loading plot (**B**) derived from the LC-IT-MS data set of *A. lentulus* and the other seven species.

Fumigati section: ▲ *A. fumigatus*, ▲ *A. fennelliae*, ▲ *A. lentulus*; *Nigri* section: ● *A. niger*, ● *A. kawachii*; *Flavi* section: ■ *A. flavus*, ■ *A. oryzae*, ■ *A. sojae*. The selected variables (VIP > 1.0 and $p < 0.05$) are highlighted in the loading plot. The alphabet means significantly different metabolites between *A. lentulus* and the other seven species and is presented in Table 2.

activity of *A. fumigatus* may have been caused by metabolites other than fumigatin.

In this study, 5,8-dihydroxy-9-octadecenoic acid (5,8-diHOE), a fatty acid derivative, has been isolated from *A. niger* [39] and was detected in section *Flavi* and in one *A. niger* strain, KCCM 60318. The biological activity of 5,8-diHOE has not yet been established; nevertheless, the effects of 5,8-diHOE are predictable based on previous reports of the antifungal [16] and anticancer [35] properties of fatty acids. However, our data suggested that this

Table 2. Tentative identification of significantly different metabolites in *A. lentulus* and the other seven species.

No.	Tentative identification	LC-IT-MS/MS						UPLC-Q-TOF-MS		Ref.
		tR (min)	*m/z* (-)	*m/z* (+)	M.W.	UV λ_{max} (nm)	$[M-H]^-$ MS^n fragment ions (m/z)	Measured $[M-H]^-$	Error (mDa)	
A	N.I.	8.7	323	325	324	211, 299	323>169,127>99>81	323.1135	-	-
B	N.I.	8.9	211	235	212	211, 300	211>123>81	211.0594	-	-
C	4,6-Dihydroxymellein	10.4	209	211	210	222, 268	209>165>123	209.0450	-0.8	[3]
D	Fumigatin	11.4	167	105	168	222, 280	167>152>108	167.0343	-0.7	[12]
E	N.I.	12.2	251	189	252	222, 283	251>233>189,161,147	-	-	-
F	N.I.	14.1	317	331	318	223	317>285>241,197	317.1060	-	-
G	N.I.	15.4	313	315	314	223	313>269>225	313.2388	-	-
H	N.I.	15.6	301	-	-	222	301>269>225,210,197,183	301.0712	-	-
I	5,8-DiHOE	20.4	313	337	314	222	313>295>277,257>233	313.2379	-2.9	[17], [27]
J	Cyclopiazonic acid	22.1	335	337	336	224, 280	335>180,140>83	335.1396	0.9	[24]
K	Neosartorin	25	679	681	680	223, 335	*681>621>561	679.1663	-1.6	[29]

Metabolites were tentatively identified using UV λ_{max}, MS^n, UPLC-Q-TOF-MS analysis, and references.
tR, retention time; M.W., molecular weight; N.I., not identified; Ref., Reference; *, positive mode;
5,8-diHOE; 5,8-dihydroxy-9-octadecenoic acid.

Table 3. Production of secondary metabolites from *Aspergillus* spp. using LC-IT-MS negative mode.

No.	Compounds	*Fumigati*											*Nigri*					
		A. fumigatus						*A. fennelliae*		*A. lentulus*			*A. niger*				*A. kawachii*	
		41186	41190	41191	41193	41196	41200	41125	41126	41939	41642	41681	60318	40280	6960	32005	11459	32819
A	N.I.	-	-	-	-	-	-	-	-	++	*	++	-	-	-	-	-	-
B	N.I.	-	-	-	-	-	-	-	-	++	++	++	-	-	-	-	-	-
C	4,6-Dihydroxymellein	-	-	-	-	-	-	-	-	+++	+++	+++	-	-	-	-	-	-
D	Fumigatin	++	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
E	N.I.	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
F	N.I.	-	+	-	-	-	-	-	-	+++	+++	+++	-	-	-	-	-	-
G	N.I.	+++	+++	++	+	+++	++	-	-	+	++	+++	-	-	-	-	-	-
H	N.I.	-	-	-	-	-	+	-	-	+++	++	+	-	-	-	-	-	-
I	5,8-DiHOE	-	-	-	-	-	-	-	-	+	+	+	-	++	-	-	-	-
J	Cyclopiazonic acid	-	-	-	-	-	-	-	-	++	++	*	-	-	-	-	-	-
K	Neosartorin	-	-	-	-	-	-	-	-	+	++	+++	-	-	-	-	-	-

No.	Compounds	*Flavi*										
		A. flavus			*A. oryzae*				*A. sojae*			
		16682	40232	6984	11896	40067	40242	40234	60354	6376	41867	40072
A	N.I.	-	-	-	-	-	-	-	-	-	-	-
B	N.I.	-	-	-	-	-	-	-	-	-	-	-
C	4,6-Dihydroxymellein	-	-	-	-	-	-	-	-	-	-	-
D	Fumigatin	-	-	-	-	-	-	-	-	-	-	-
E	N.I.	-	-	-	-	-	-	-	-	-	-	-
F	N.I.	-	-	-	-	-	-	-	-	-	-	-
G	N.I.	-	-	-	-	-	-	-	-	-	-	-
H	N.I.	-	-	-	-	-	-	-	-	-	-	-
I	5,8-DiHOE	+	*	*	+	+	*	*	+	+	++	+
J	Cyclopiazonic acid	-	-	-	-	-	-	-	-	-	-	-
K	Neosartorin	-	-	-	-	-	-	-	-	-	-	-

The production of each metabolite was calculated by the peak intensities from an LC-IT-MS chromatogram

*: < 0.5 k count, +: > 0.5 k count, ++: > 5 k count, +++: > 10 k count, -; not detected.

5,8-diHOE; 5,8-dihydroxy-9-octadecenoic acid.

metabolite did not possess any antibacterial activity against *S. epidermidis*.

Cyclopiazonic acid is a toxic indole tetramic acid, first isolated from *Penicillium* and has been previously reported as a well-known mycotoxin in *A. lentulus* [22]. Several studies have shown that cyclopiazonic acid is produced by various *Aspergillus* species such as *A. flavus A. oryzae*, and *A. lentulus* [6]. Our study also showed that this metabolite is not produced by *A. flavus* and *A. oryzae*. Cvetnic and Pepeljnjak [7] earlier showed that all strains of *A. flavus* did not produce cyclopiazonic acid. It may be possible that secondary metabolite production is influenced by culture conditions (i.e., medium, pH, and incubation temperature) [10, 31, 43].

In our current study, neosartorin was detected as a significantly different metabolite between *A. lentulus* and the other seven species. As one of the mycotoxins similar to cyclopiazonic acid, neosartorin was isolated from the mycelium of *Neosartorya fischeri* in 1998 and possesses a hydroxyl hydrogen bonded to a carbonyl group, with carboxymethyl and acetoxy carbonyls attached to a benzene ring. This study also showed the antibacterial effect of neosartorin against *S. epidermidis*. This metabolite is one of the asymmetric ergochromes, which are a group of light

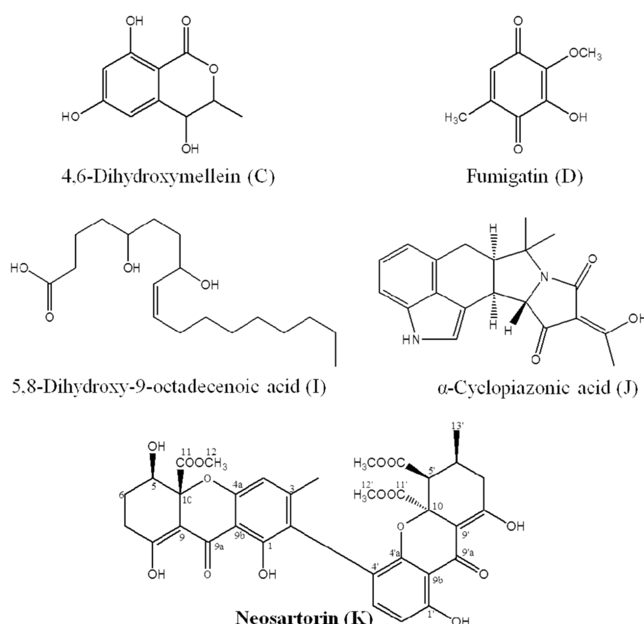

Fig. 3. The structures of significantly different metabolites between *A. lentulus* and the other seven species.

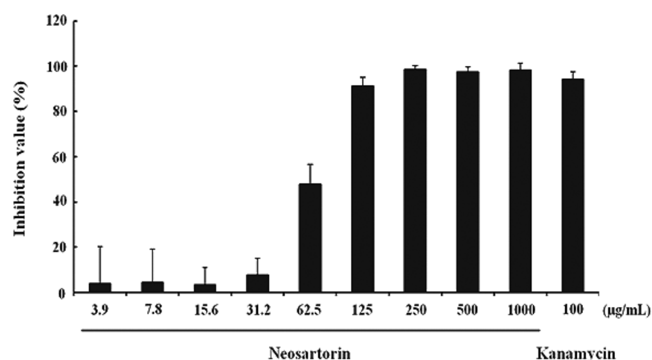

Fig. 4. Antibacterial activity of neosartorin isolated from *A. lentulus*.

Inhibition rates of neosartorin against *Staphylococcus epidermidis* were determined by MIC. Kanamycin (100 μg/ml) was used as the positive control.

yellow mycotoxins that include secalonic acid or eumitrin A_1 [29]. Diverse biological activities of secalonic acids have earlier been reported, including toxicity, antifungal, and allelopathic effects [20, 33, 44]. However, the study on the structural relationship of neosartorin to secalonic acids and eumitrin A_1 remains elusive. Therefore, we investigated the inhibitory effect of neosartorin on cell growth in A549 human non-small lung carcinoma cells. Our results showed that neosartorin possessed anticancer activity, suppressing cell proliferation at an effective concentration of 10 μM. To

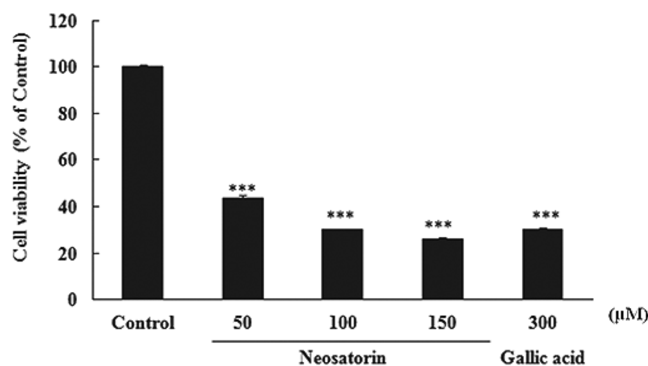

Fig. 5. Inhibitory effects of neosartorin isolated from *A. lentulus* on the cell viability of A549 cells.

Cell viability was evaluated after neosartorin treatment at the concentrations ranging from 50 to 150 μM. Neosartorin showed an inhibition of cell proliferation in a dose-dependent manner. Each value represents the mean ± SE of three experiments. * indicates the significance at $p < 0.05$ compared with control cells (***$p < 0.001$). Gallic acid (300 μM) was used as the positive control.

our knowledge, this is the first report that describes the anticancer and antibacterial effects of neosartorin.

In this study, *Aspergillus* species were classified according to secondary metabolite profiling using a LC-IT-MS-based metabolomics technique, resulting in the identification of five significantly different metabolites between *A. lentulus* and the other seven species. In addition, *A. lentulus* and its active compound, neosartorin, suppressed the bacterial growth in *S. epidermidis*. This result revealed that neosartorin was either the discriminable metabolite between *Aspergillus* species, or an antibacterial agent from *A. lentulus*. This approach also suggests that a secondary metabolite-based chemotaxonomic analysis may serve as a useful tool for conventional classification, as well as for evaluation of species-specific bioactivity.

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References

1. Arora DK. 2003. *Fungal Biotechnology in Agricultural, Food, and Environmental Applications*, pp. 51-53. Marcel Dekker, Inc., New York, USA.
2. Arora DS, Chandra P. 2011. Antioxidant activity of *Aspergillus fumigatus*. *ISRN Pharmacol.* **2011:** 1-11.

3. Avantaggiato G, Solfrizzo M, Tosi L, Zazzerini A, Fanizzi FP, Visconti A. 1999. Isolation and characterization of phytotoxic compounds produced by *Phomopsis helianthi*. *Nat. Toxins* **7:** 119-127.
4. Bennett JW, Klich M. 2003. Mycotoxins. *Clin. Microbiol. Rev.* **16:** 497-516.
5. Carolis DE, Posteraro B, Lass-Florl C, Vella A, Florio AR, Torelli R, *et al*. 2012. Species identification of *Aspergillus, Fusarium* and *Mucorales* with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Microbiol. Infect*. **18:** 475-484.
6. Chang PK, Ehrlich KC, Fujii I. 2009. Cyclopiazonic acid biosynthesis of *Aspergillus flavus* and *Aspergillus oryzae*. *Toxins* **1:** 74-99.
7. Cvetnic Z, Pepeljnjak S. 1998. Production of cyclopiazonic acid by aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavu. Nahrung* **42:** 321-323.
8. Dunn WB, Ellis DI. 2005. Metabolomics: current analytical platforms and methodologies. *Trends Anal. Chem*. **24:** 285-294.
9. Elaasser MM, Abdel-Aziz MM, EI-Kassas RA. 2011. Antioxidant, antimicrobial, antiviral and antitumor activities of pyranone derivative obtained from *Aspergillus candidus*. *J. Microbiol. Biotechnol. Res*. **1:** 5-17.
10. Elias BC, Said S, de Albuquerque S, Pupo MT. 2006. The influence of culture conditions on the biosynthesis of secondary metabolites by *Penicillium verrucosum* Dierck. *Microbiol. Res*. **161:** 273-280.
11. Espinel-Ingroff A, Arthington-Skaggs B, Iqbal N, Ellis D, Pfaller MA, Messer S, *et al*. 2007. Multicenter evaluation of a new disk agar diffusion method for susceptibility testing of filamentous fungi with voriconazole, posaconazole, itraconazole, amphotericin B, and caspofungin. *J. Clin. Microbiol*. **45:** 1811-1820.
12. Frisvad JC, Rank C, Nielsen NF, Larsen TO. 2009. Metabolomics of *Aspergillus fumigatus. Med. Mycol.* **47:** 53-71.
13. Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA. 2007. The current status of species recognition and identification in *Aspergillus. Stud. Mycol.* **59:** 1-10.
14. Goker H, Ozden S, Yildiz S, Boykin DW. 2005. Synthesis and potent antibacterial activity against MRSA of some novel 1,2-disubstituted-1*H*-benzimidazole-*N*-alkylated-5-carboxamidines. *Eur. J. Med. Chem*. **40:** 1062-1069.
15. Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA. 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* **97:** 1316-1329.
16. Kabara JJ, Swieczkowski DM, Conley AJ, Truant JP. 1972. Fatty acids and derivatives as antimicrobial agents. *Antimicrob. Agents Chemother.* **2:** 23-28.
17. Kang DJ, Son GH, Park HM, Kim JY, Choi JN, Kim HY, *et al*. 2013. Culture condition-dependent metabolite profiling of *Aspergillus fumigatus* with antifungal activity. *Fungal Biol*. **117:** 211-219.
18. Kang DJ, Kim JY, Choi JN, Liu KH, Lee CH. 2011. Chemotaxonomy of *Trichoderma* spp. using mass spectrometry-based metabolite profiling. *J. Microbiol. Biotechnol.* **21:** 5-13.
19. Kim HY, Park HM, Lee CH. 2012. Mass spectrometry-based chemotaxonomic classification of *Penicillium* species (*P. echinulatum*, *P. expansum*, *P. solitum*, and *P. oxalicum*) and its correlation with antioxidant activity. *J. Microbiol. Methods* **90:** 327-335.
20. Kurobane I, Iwahashi S, Fukuda A. 1987. Cytostatic activity of naturally isolated isomers of secalonic acids and their chemically rearranged dimers. *Drugs Exp. Clin. Res.* **13:** 339-344.
21. Lamrani K, Lakhtar H, Ismaili-Alaoui M, Ettalibi M, Boiron P, Augur C, *et al*. 2008. Production of Fumagillin by *Aspergillus fumigatus* isolated from traditional trituration units, “Maasra,” in Morocco. *Micol. Apl. Int.* **20:** 35-41.
22. Larsen TO, Smedsgaard J, Nielsen KF, Hansen MA, Samson RA, Frisvad JC. 2007. Production of mycotoxins by *Aspergillus lentulus* and other medically important and closely related species in section *Fumigati. Med. Mycol.* **45:** 225-232.
23. Lommen A. 2009. Metalign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Anal. Chem.* **81:** 3079-3086.
24. Losito I, Monaci L, Aresta A, Zambonin CG. 2002. LC-ion trap electrospray MS-MS for the determination of cyclopiazonic acid in milk samples. *Analyst* **127:** 499-502.
25. Mabe S, Eller J, Champney WS. 2004. Structure-activity relationships for three macrolide antibiotics in *Haemophilus influenzae. Curr. Microbiol.* **49:** 248-254.
26. Machida M, Gomi K. 2010. *Aspergillus: Molecular Biology and Genomics*, pp. 8-9. Caister Academic Press, Norfolk, UK.
27. Mazur P, Nakanishi K, El-Zayat AAE, Champe SP. 1991. Structure and synthesis of sporogenic psi factors from *Aspergillus nidulans*. *J. Chem. Soc. Chem. Commun.* **20:** 1486–1487.
28. Polizzi V, Adams A, Malysheva SV, Saeger SD, Peteghem CV, Moretti A, *et al*. 2012. Identification of volatile markers for indoor fungal growth and chemotaxonomic classification of *Aspergillus* species. *Fungal Biol*. **116:** 941-953.
29. Proksa B, Uhrin D, Liptaj T, Sturdikova M. 1998. Neosartorin, an ergochrome biosynthesized by *Neosatorya fischeri*. *Phytochemistry* **48:** 1161-1164.
30. Rai M, Kovics G. 2010. *Progress in Mycology*, pp. 95-97. Scientific Publishers, Jodhpur, India.
31. Ramos HP, Said S. 2011. Modulation of biological activities produced by an endophytic fungus under different culture conditions. *Adv. Biosci. Biotechnol*. **2:** 443-449.
32. Rank C, Klejnstrup ML, Petersen LM, Kildgaard S, Frisvad JC, Gotfredsen CH, *et al*. 2012. Comparative chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL 3357). *Metabolites* **2:** 39-56.
33. Ren H, Tian L, Gu Q, Zhu W. 2006. Secalonic acid D; a cytotoxic constituent from marine lichen-derived fungus *Gliocladium* sp. T31. *Arch. Pharm. Res.* **29:** 59-63.

34. Rodrigues P, Santos C, Venancio A, Lima N. 2011. Species identification of *Aspergillus* section *Flavi* isolates from Portuguese almonds using phenotypic, including MALDI-TOF-ICMS and molecular approaches. *J. Appl. Microbiol*. **111:** 877-892.
35. Siegel I, Liu TL, Yaghoubzadeh E, Keskey TS, Gleicher N. 1987. Cytotoxic effects of free fatty acids on ascites tumor cells. *J. Natl. Cancer Inst.* **78:** 271-277.
36. Sun HF, Li XM, Meng L, Cui CM, Gao SS, Li CS, *et al*. 2012. Asperolides A-C, tetranorlabdane diterpenoids from the marine alga-derived endophytic fungus *Aspergillus wenti*i EN-48. *J. Nat. Prod.* **75:** 148-152.
37. Turner NW, Subrahmanyam S, Piletsky SA. 2009. Analytical methods for determination of mycotoxins: a review. *Anal. Chim. Acta* **632:** 168-180.
38. Varga J, Rigo K, Toth B, Teren J, Jozakiewicz Z. 2003. Evolutionary relationships among *Aspergillus* species producing economically important mycotoxins. *Food Technol. Biotechnol.* **41:** 29-36.
39. Wadman MW, de Vries RP, Kalkhove SIC, Vel-dink GA, Vliegenthart JFG. 2009. Characterization of oxylipins and dioxygenase genes in the asexual fungus *Aspergillus niger*. *BMC Microbiol*. **9:** 59.
40. Waksman SA, Horning ES, Spencer EL. 1943. Two antagonistic fungi, *Aspergillus fumigatus* and *Aspergillus clavatus*, and their antibiotic substances. *J. Bacteriol*. **45:** 233-248.
41. Wang Y, Zheng J, Liu P, Wang W, Zhu W. 2011. Three new compounds from *Aspergillus terreus* PT06-2 grown in a high salt medium. *Mar. Drugs* **9:** 1368-1378.
42. Yen GC, Chang YC, Su SW. 2003. Antioxidant activity and active compounds of rice *koji* fermented with *Aspergillus candidus*. *Food Chem*. **83:** 49-54.
43. Zain ME, Razak AA, EI-Sheikh HH, Soliman HG, Khalil AM. 2009. Influence of growth medium on diagnostic characters of *Aspergillus* and *Penicillium* species. *Afr. J. Microbiol. Res.* **3:** 280-286.
44. Zeng RS, Luo SM, Shi YH, Shi MB, Tu CY. 2001. Physiological and biochemical mechanism of allelopathy of secalonic acid F on higher plants. *Agron. J*. **93:** 72-79.