# Isolation and Characterization of Antioxidant Compounds from *Aspergillus candidus* Broth Filtrate

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The objectives of this study were to isolate the antioxidative components in the broth filtrate of *Aspergillus candidus* (CCRC 31543), to characterize their antioxidative properties, and to evaluate their safety. Three major compounds were isolated and identified as 3,3″-dihydroxyterphenyllin, 3-hydroxyterphenyllin, and candidusin B. In the linoleic acid peroxidation system, the inhibition of peroxidation in these three compounds was greater than 95% and was significantly higher than that of α-tocopherol but equal to that of BHA at 12.5−200 μg/mL. As measured using the Rancimat method in lard, 3,3″-di-OH-terphenyllin exhibited a protection factor value of 7.82, which was substantially higher than those of BHA (5.58) and α-tocopherol (4.29) at 200 μg/mL. 3,3″-di-OH-terphenyllin and 3-OH-terphenyllin also exhibited marked scavenging effects on the α,α-diphenyl-β-picrylhydrazyl radicals (94.7 and 96.0%, respectively), which were similar to those of BHA and α-tocopherol. Safety studies showed that these three compounds were neither cyto- nor geno-toxic toward human intestine 407 (INT 407) cells, nor mutagenic toward *Salmonella typhimurium* TA98 and TA100.

**Keywords:** *Aspergillus candidus; antioxidant; Rancimat; free radical scavenging; safety*

## INTRODUCTION

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ), are usually used as food additives by the food industry to prevent lipid peroxidation. However, their application has been reassessed because of possible toxic and carcinogenic components formed during their degradation (*1, 2*). Because of these health concerns, natural antioxidants, such as α-tocopherol and ascorbic acid, have been extensively employed in recent years. Compared to synthetic compounds, natural antioxidants are usually more expensive but inferior in effect. Thus, finding safer and more effective and economic natural antioxidants is highly desirable. Microorganisms have been shown to be a potential source of natural antioxidants (*3*). *Penicillium roquefortii* IFO 5956 (*4*) and *Mortierella* sp. fungus (*5*) have been reported to produce antioxidants as metabolites.

In our previous studies, *Aspergillus candidus* (CCRC 31543) was screened from 10 other molds, and its broth extract was found to exhibit the highest antioxidative activity (*6*). The antioxidant properties of the ethyl acetate extracts from *A. candidus* broth filtrate were evaluated using various model systems, and it was found that this extract might be a potential antioxidant for application in food products (*7*). The production of extract had also been optimized (*8, 9*), and was found to show no mutagenicity as evaluated using the Ames test (*7*). The objective of this current study was to isolate and identify the antioxidative components in the ethyl acetate extract. The antioxidative properties of these *Aspergillus* metabolites were evaluated and compared to those of BHA and α-tocopherol using various methods. Moreover, safety studies were also made to evaluate the cytotoxicity, genotoxicity, and mutagenicity of the isolated compounds.

## MATERIALS AND METHODS

**Microorganism and Cells.** *Aspergillus candidus* (CCRC 31543) and human intestine 407 (INT 407) cells (CCRC 60022) were obtained from the Culture Collection and Research Center (CCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). The histidine-requiring strains of *Salmonella typhimurium* strains TA98 and TA100 were kindly supplied by Dr. B. N. Ames (University of California, Berkeley, CA). The stock culture of *A. candidus* was grown on the slant of potato dextrose agar (PDA) and maintained at 25 °C. Spore suspension of *A. candidus* was prepared in sterile water and used for inoculation.

**Medium and Chemicals.** The medium used to produce antioxidants was prepared according to the modified formula of Aoyama (*10*) and consisted of 3% sucrose, 0.1% yeast extract, 0.1% polypeptone, 0.3% $(NH_4)SO_4$, 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4 \cdot 7H_2O$. INT 407 cells were grown in a 25T flask (Nunc, Inc., Naperville, IL) using Basal Medium Eagle with Hank's salts supplemented with 15% bovine calf serum, 2 mM L-glutamine, 50 U/mL penicillin G, 50 μg/mL streptomycin, and 131 mM $Na_2HCO_3$ as growth medium.

Yeast extract was purchased from Difco Co. (Detriot, MI). Polypeptone was obtained from Nihon Seiyaku Co. (Osaka, Japan). $(NH_4)SO_4$ and $K_2HPO_4$ were purchased from Ishizu Pharmaceuticals (Osaka, Japan), and $MgSO_4 \cdot 7H_2O$ was the product of Junsei Chemicals (Tokyo, Japan). KCl and $FeSO_4 \cdot 7H_2O$ were obtained from Wako Pure Chemicals (Osaka, Japan) and Hayashi Pure Chemicals (Osaka, Japan), respectively. Acetone and *n*-hexane used for column chromatography were obtained from J. T. Baker, Inc. (Phillipsburg, NJ). Ferrous chloride, ammonium thiocyanate, and silica gel 60 (70−230 mesh) were purchased from E. Merck (Darmstadt, Germany). BHA and α-tocopherol were obtained from Sigma

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Chemical Co. (St. Louis, MO). Lard without any antioxidant added was obtained from Chian-Kwuan Oil Co. (Kaohsiung, Taiwan). Basal Medium Eagle with Hank's salts, bovine calf serum, and antibiotics containing penicillin G/streptomycin were purchased from Gibco/BRL Life Technologies (Eggenstein, Germany).

**Culture Conditions.** Culture conditions followed the method of Yen and Lee (*6*). Spore suspension (5 mL, $10^7$ spores/mL) of *A. candidus* (CCRC 31543) was inoculated into a 1000-mL Hilton flask containing 300 mL of medium. The flasks were incubated at 25 °C by shaking at 100 rpm for 48 h on a rotary shaker (model S302A, Firstek orbital shaker). This inoculum (300 mL) was then transferred into 3 L of medium (inoculum of 10%, v/v) and incubated under the same conditions for 24 h. The broth (3 L) was transferred into a 100-L fermentor (Mitsuwa model KMJ-100 MST-FMPC-MII) containing 70 L of the same medium. Fermentation was conducted at 25 °C with airflow (0.5 L/min) and agitation (100 rpm) for 13 days.

**Extraction and Isolation of Antioxidant Substances from the Broth Filtrate of *A. candidus*.** The broth filtrate (70 L) of *A. candidus* was separated from the mycelia by filtering through a Whatman No. 1 filter paper under suction, and an equal volume of ethyl acetate was added for extraction. The mixture was shaken in a separatory funnel for 20 min. The organic layer was collected, dried over anhydrous sodium sulfate (1%, w/v), filtered, evaporated to dryness in vacuo, and weighed to determine the yields of soluble solids.

The ethyl acetate (EtoAc) soluble material (10.07 g) was chromatographed on a silica gel column (850 × 50 mm i.d.) with an acetone/*n*-hexane gradient (20:80 to 50:50 in a total volume of 41 L). Fractions SG-I−SG-IX were collected in different volumes of solvents (3, 5, 5, 4, 4, 8, 4, 4, and 4 L, respectively), evaporated in vacuo, and weighed to determine the yields. The components of each fraction (SG-I−SG-IX) were analyzed by high-performance liquid chromatography (HPLC). The fractions were further fractionated by a preparative HPLC using a Mightysil RP-18 reversed-phase column (5 μm, 250 × 20 mm i.d., Kanto Chemical Co.) in the same mobile phase system at a flow rate of 6.0 mL/min and an injection volume of 0.3−0.5 mL. Subfractions were then analyzed to evaluate their purity by means of HPLC.

**HPLC Analyses.** For HPLC analyses, each dry fraction and subfraction sample was dissolved in methanol (0.5 mL) and filtered through a 0.45-μm filter, and 10 μL of each filtrate was injected into the HPLC system. The HPLC apparatus was a Hitachi liquid chromatograph (Hitachi Ltd.), consisting of a model L-6200 intelligent pump, a Rheodyne model 7125 syringe-loading sample injector, a model D-2000 integrator, and a model L-4200 UV−vis detector set at 280 nm. The column was a LiChrosorb 100 RP-18 (5 μm, 250 × 4.6 mm i.d.; E. Merck). The solvent system used was a gradient of solvent A (acetonitrile) and solvent B (aqueous trifluoroacetic acid, 0.1%, v/v). The following gradient was applied: 0 min, 75% B; 0−15 min, 60% B linear; 15−28 min, 75% B linear; 28−30 min 75% B isocratic, followed by washing and reconditioning of the column. The flow rate was set at 1.0 mL/min.

**Spectrometry.** The UV−vis absorption spectra of the active components in methanol were recorded on a Hitachi U-3000 spectrophotometer. The mass spectra of the components were obtained using the fast atom bombardment (FAB) ionization mode (NBA matrix) on a JEOL JMS-SX/SX 102A mass spectrometer. The IR spectra of the samples in KBr pellets were obtained using a Bruker Equinox 55 infrared spectrophotometer.

The nuclear magnetic resonance (NMR) spectra were measured in acetone-$d_6$ with a Varian VXR-300s FT-NMR spectrometer operating at 299.95 MHz for $^1$H NMR and 75.43 MHz for $^{13}$C NMR with complete proton decoupling. The sweep width, pulse angle, repetition delay, and acquisition time for $^1$H NMR were 4500.0 Hz, 7.0 μs, 0 and 2.0 s, respectively, and for $^{13}$C NMR were 25000.0 Hz, 7.0 μs, 2.0 and 1.0 s, respectively. The chemical shifts are reported in parts per million (ppm) from tetramethylsilane.

**Antioxidant Activity Determined using the Thiocyanate Method.** The antioxidant activity of inhibiting linoleic acid peroxidation was assayed using the thiocyanate method (*11*). A 0.5 mL aliquot of methanol solution (125−2000 μg/mL) of the isolated compounds was mixed with linoleic acid emulsion (2.5 mL, 0.02 M, pH 7.0) and phosphate buffer (2 mL, 0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as emulsifer, and 50 mL of phosphate buffer, and then the mixture was homogenized. The reaction mixture was incubated at 37 °C to accelerate oxidation. The levels of oxidation were determined by measuring the absorbance at 500 nm with a Hitachi U-2000 spectrophotometer after reaction with ferrous chloride and ammonium thiocyanate. The antioxidant activity was expressed as a percentage of inhibition of peroxidation (IP%): IP% = [1 − (absorbance of sample at 500 nm)/(absorbance of control at 500 nm) ] × 100. The antioxidant activities of BHA and α-tocopherol were also assayed for comparison purposes. All the tests were performed in triplicate and averaged.

**Antioxidant Activity Determined using the Rancimat Method.** Lard was used as the lipid substrate to evaluate the lipid oxidation inhibition activity of EtoAc extract, the isolated compounds, and BHA. A Metrohm 679 Rancimat instrument was used in this experiment. Aliquots (0.5 mL) of methanol solution (125−1000 μg/mL) of EtoAc extract or the isolated compounds from EtoAc extract were mixed with 2.5 g of lard in glass cylinders. The oxidation experiments were carried out at 100 °C, and air was blown through at a flow rate of 20 mL/min (*12*). For the control experiment, 0.5 mL of methanol was added to 2.5 g of lard, and the experiment was conducted under the same conditions as described above. The inhibitory activities of BHA and α-tocopherol dissolved in methanol (1000 μg/mL) were also analyzed for comparison purposes. All the tests were performed in triplicate and averaged.

**Scavenging Effect on α,α-Diphenyl-β-picrylhydrazyl (DPPH) Radicals.** The scavenging effect of the isolated compounds on the DPPH radicals was estimated according to the method of Hatano et al. (*13*). Each sample (0.5 mg) in 4 mL of methanol was mixed with 1.0 mL of a solution of DPPH radical in methanol. The mixture was shaken vigorously and kept for 30 min, and the absorbance of the mixture at 517 nm was measured using a Hitachi U-2000 spectrophotometer. All the tests and analyses were performed in triplicate and averaged.

**Cytotoxicity Analysis.** To measure the acute cytotoxicity, 0.49 mL of each original cell suspension was mixed with 10 mL of a 0.4% trypan blue solution and checked for viability 5 min later. The cells were analyzed through microscopic observation, and the percentage of viable cells was determined.

**Mutagenicity Assay.** The mutagenic effects of the identified compounds were assayed according to the Ames test using *Salmonella typhimurium* strains TA98 and TA100 (*14*). The $His^+$ revertant colonies were counted after incubation at 37 °C for 48 h. The experiment was performed at least twice. Each sample was assayed in triplicate plates per run and data presented are means ± SD. In this mutagenicity test, the result was recognized as positive when the number exceeded twice the number of spontaneous revertant.

**Alkaline Single-Cell Gel Electrophoresis (Comet Assay).** The comet assay was performed under alkaline conditions following the methods of Yen et al. (*15*). The slides were observed using a fluorescent microscope attached to a CCD camera and connected to a personal-computer-based image analysis system (Komet 3.0; Kinetic Imaging Ltd.). For each analysis, 50 individual cells were calculated, and in most cases three separate experiments were conducted for each series. Single cells were analyzed under the fluorescent microscope as desired. The DNA damage was expressed as tail moment, where tail moment = [tail length × tail DNA/(head DNA + tail DNA)] × 100. A higher tail moment meant a higher level of DNA damage.

**Statistical Analysis.** Statistical analyses were performed according to the SAS User's Guide. Analysis of variance was performed using the ANOVA procedure. Significant differences ($p < 0.05$) between means were determined using Duncan's multiple range tests.

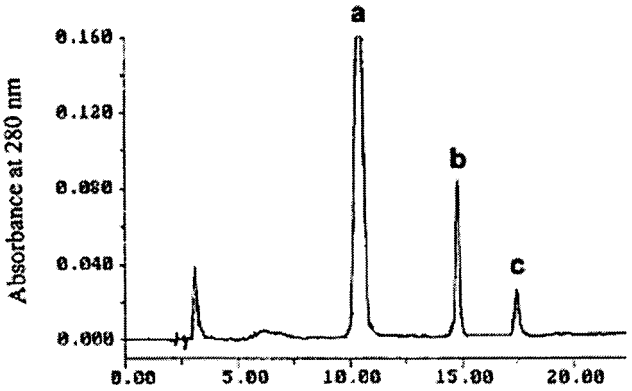


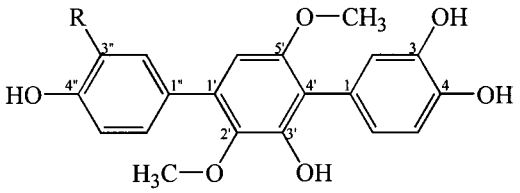

**Figure 1.** HPLC chromatograms of ethyl acetate extracts from *A. candidus* (CCRC 31543) broth filtrate. The retention times of compounds **a**, **b**, and **c** are 10.8, 14.8, and 17.1 min, respectively.

## RESULTS AND DICUSSION

**Yields of Antioxidant Components from Ethyl Acetate Extract.** A pilot-scale fermentation of *A. candidus* was made to obtain sufficient quantities of antioxidative components. After 13 days of fermentation at 25 °C, 4457.7 g of EtoAc soluble material was extracted from 70 L of the broth filtrate. The total yield was 6.37%. From the HPLC chromatogram of this material (Figure 1), three major components, **a**, **b**, and **c**, were found with retention times of 10.8, 14.8, and 17.1 min, respectively. The yields of components **a**, **b**, and **c** were determined to be 13.2, 2.2, and 2.3 mg/mL of the broth filtrate, respectively. The overall content of components **a**, **b**, and **c** in EtoAc extract was 27.8%.

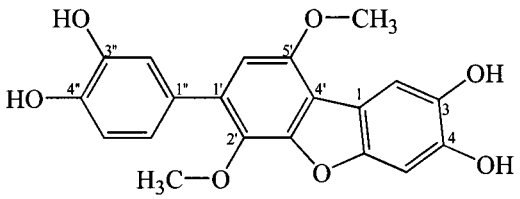
**Isolation and Identification of Antioxidant Components from Ethyl Acetate Extract.** The major components in the EtoAc extract were isolated by means of silica gel column chromatography and preparative HPLC. With column chromatographic separation on silica gel, the extract was separated into nine fractions (**SG-I**− **SG-IX**). The composition of components **a**, **b**, and **c** in each fraction was measured by HPLC analysis. Among the 9 fractions, the **SG-V** and **SG-VI** fractions possessed the highest yields (14.59 and 33.30%, respectively) and contained the major components **a**, **b**, and **c**. Both fractions were individually collected for further purification. Preparative HPLC separation of **SG-V** and **SG-VI** individually afforded three subfractions of components **a**, **b**, and **c,** which was confirmed by analytical HPLC. The retention times of purified components **a**, **b**, and **c** and were found to correspond to those in the EtoAc extract chromatogram (Figure 1).

Subfractions of compound **a**, **b,** and **c** were collected from **SG-VI** and **SG-V**. Compounds **a**, **b**, and **c** were recrystallized from acetone/*n*-hexane (40:60, 35:65, and 35:65, v/v, respectively) to yield pale yellow prisms. From UV−Vis, IR, MS, $^{1}$H NMR, and $^{13}$C NMR data, compounds **a**, **b**, and **c** were identified as 3,3″-dihydroxyterphenyllin (2′,5′-dimethoxy-3,3″,3″,4,4′-pentahydroxy-*p*-terphenyl), 3-hydroxyterphenyllin (2′,5′-dimethoxy-3,4,3′,4″-tetrahydroxy-*p*-terphenyl), and candidusin B (2,2′-epoxy-3′,6′-dimethoxy-1,1′:4′,1″-terphenyl-3″,4,4″,5-tetrol), respectively. The spectral characteristics of compounds **a**, **b**, and **c** were identical to those reported by Kobayashi et al. (*16*), Kurobane et al. (*17*), and Kobayashi et al. (*18*) as *A. candidus* metabolites. The structures of the three compounds are shown in Figure 2.




Compound **a**: 3,3”-dihydroxyterphenyllin, R = OH

Compound **b**: 3-hydroxyterphenyllin, R = H




Compound **c**: candidusin B

**Figure 2.** Structures of antioxidant compounds **a**, **b**, and **c** isolated from the ethyl acetate extracts of *A. candidus* (CCRC 31543) broth filtrate.

**Inhibition of Linoleic Acid Peroxidation.** The antioxidant activities of 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B were determined using the thiocyanate method and are shown in Table 1. These compounds exhibited above 95% inhibition of linoleic acid peroxidation (IP%) at 12.5−200 $\mu$g/mL except for 3,3″-di-OH terphenyllin (93.5%), which required a concentration of 200 $\mu$g/mL. However, the IP% of 3,3″-di-OH terphenyllin decreased with increasing concentration within a concentration range of 50−200 $\mu$g/mL, which indicates that 3,3″-di-OH terphenyllin exhibits strong antioxidant activity at lower concentrations (12.5−100 $\mu$g/mL). At 200 $\mu$g/mL, the IP% of these compounds were significantly higher than those of α-tocopherol (88.9%) and EtoAc extract (92.0%), but close to that of BHA (98.8%).

It has been reported that most antioxidants have phenolic hydroxyl groups in their structures (*19*−*21*), and that the antioxidant activity of monophenols might be improved substantially by the addition of a methoxy substituent *ortho*- to the hydroxyl group (*22*, *23*). The structures of 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B possess a number of phenolic hydroxyl groups and an *ortho*-methoxy substitution relative to the hydroxyl group in the central aromatic ring. The strong antioxidant activities of these compounds are believed to be a result of these hydroxyl groups and the *ortho*-methoxy substituent in their chemical structures.

**Prevention of Lard Oxidation.** The Rancimat method is commonly used to evaluate the antioxidative potency of various antioxidants (*24*) and is based on the increase of electrical conductivity due to the formation of volatile dicarboxylic acids as a result of lipid oxidation (*25*). The addition of 3,3″-di-OH terphenyllin to lard markedly extended the induction time of lipid oxidation (Table 2). The induction time of 3,3″-di-OH terphenyllin increased with increasing concentration and was significantly ($p < 0.05$) higher than those of 3-OH terphenyllin and candidusin B within the 25−200 $\mu$g/mL

**Table 1. Inhibition of Linoleic Acid Peroxidation (IP%) of 3,3″-Dihydroxyterphenyllin, 3-Hydroxyterphenyllin, and Candidusin B as Measured by the Thiocyanate Method**

| | inhibition of peroxidation (%)[a] | | | | |
|---|---|---|---|---|---|
| | 12.5 μg/mL | 25 μg/mL | 50 μg/mL | 100 μg/mL | 200 μg/mL |
| 3,3″-di-OH terphyllin | 99.3 ± 0.3[ab] | 98.7 ± 0.9[a] | 98.3 ± 1.0[a] | 95.4 ± 0.3[b] | 93.5 ± 1.4[c] |
| 3-OH terphyllin | 99.2 ± 0.5[a] | 99.1 ± 0.1[a] | 99.3 ± 0.7[a] | 98.2 ± 0.7[a] | 98.5 ± 0.3[a] |
| candidusin B | 99.1 ± 0.3[a] | 99.1 ± 0.2[a] | 98.6 ± 0.0[a] | 99.1 ± 0.1[a] | 98.6 ± 1.1[a] |
| EtoAc extract | | | | | 92.2 ± 0.2[d] |
| BHA | | | | | 98.8 ± 0.5[a] |
| α-tocopherol | | | | | 89.0 ± 1.0[e] |

[a] Inhibition of peroxidation (%) = [1 − (absorbance of sample at 500 nm/absorbance of control at 500 nm)] × 100. [b] Each value is the mean ± SD ($n = 3$). Values in each column with different superscripts are significantly different ($p < 0.05$).

**Table 2. Prevention of Lard Oxidation by the Isolated Compounds 3,3″-Dihydroxyterphenyllin, 3-Hydroxyterphenyllin, and Candidusin B as Measured by the Rancimat Method**

| | induction time (h) (PF)[a] | | | |
|---|---|---|---|---|
| | 25 μg/mL | 50 μg/mL | 100 μg/mL | 200 μg/mL |
| control | 1.9 ± 0.2[eb](1.0) | 1.9 ± 0.2[b] (1.0) | 1.9 ± 0.2[d] (1.0) | 1.9 ± 0.2[d] (1.0) |
| 3,3″-di-OH terphyllin | 7.4 ± 0.1[a] (3.9) | 9.4 ± 1.7[a] (5.0) | 11.5 ± 3.3[b] (6.0) | 14.9 ± 0.4[b] (7.8) |
| 3-OH terphyllin | 3.9 ± 0.0[c] (2.1) | 6.9 ± 0.9[a] (3.7) | 8.4 ± 1.8[bc](4.4) | 8.8 ± 0.2[c] (4.6) |
| candidusin B | 3.1 ± 0.0[d] (1.6) | 3.7 ± 0.0[b] (2.0) | 4.4 ± 0.0[cd](2.3) | 7.4 ± 0.2[c] (3.9) |
| EtoAc extract | 4.5 ± 0.3[b] (2.4) | 8.3 ± 0.8[a] (4.4) | 18.5 ± 2.3[a] (9.7) | 30.1 ± 4.4[a] (15.8) |
| BHA | | | | 10.6 ± 0.7[c] (5.6) |
| α-tocopherol | | | | 8.2 ± 0.1[c] (4.3) |

[a] Values in parentheses are protection factor. Protection factor (PF) = (induction time of antioxidant)/(induction time of control). [b] Reported values are the mean ± SD ($n = 3$). Data bearing different superscripts within a column are significantly different ($p < 0.05$).

range. 3,3″-di-OH Terphenyllin exhibited strong antioxidant activity with a protection factor (PF) value of 7.82, which was substantially ($p < 0.05$) higher than those of BHA (5.6) and α-tocopherol (4.3), but was lower than that of ethyl acetate extract (9.7) at 200 μg/mL. Yen and Lee (*7*) reported that the extract has a hydrogen donating ability and a synergistic effect with some known antioxidants. The higher antioxidant activity of the extract might result from the synergistic effect between it and the antioxidant components in the extract.

3,3″-di-OH terphenyllin possesses two pairs of *ortho*-substituted hydroxyl groups on its lateral aromatic rings, but 3-OH terphenyllin has only one pair on the first aromatic ring. The presence of a second hydroxyl group in the *ortho* or *para* position is known to increase the antioxidant activity because of additional resonance stabilization and *o*-quinone or *p*-quinone formation (*22*, *26*, *27*). This may be the reason 3,3″-di-OH terphenyllin has better antioxidant activity than the other compounds.

**Scavenging DPPH Radical.** The DPPH radical is considered to be a model compound of a lipophilic radical. Lipid autoxidation initiates a chain reaction in lipophilic radicals. The scavenging effects of 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B on DPPH radical are shown in Table 3. Both 3,3″-di-OH terphenyllin and 3-OH terphenyllin were found to have good scavenging effects (94.7 and 96.0%, respectively) on the DPPH radicals. There were no significant ($p > 0.05$) differences among the scavenging effects of 3,3″-di-OH terphenyllin, 3-OH terphenyllin, BHA, and α-tocopherol. However, lower scavenging effects were found for EtoAc extract (84.4%) and candidusin B (59.2%). The activity of the compounds corresponds to the number of hydrogens available for donation by hydroxyl groups (*28*). For example, rosmarinic acid has four hydroxyl groups that can reduce four DPPH radicals and, therefore, exhibits a major scavenging effect (*27*). By the same token, the radical scavenging effects of 3,3″-di-OH terphenyllin and 3-OH terphenyllin may be due to these hydroxyl groups. Moreover, the *ortho*-methoxy group on their central aromatic rings also stabilizes the aryloxy radical by electron donation, thereby increasing the antioxidant and antiradical efficiencies. The lower scavenging effect of candidusin B might result from the absence of an *ortho*-methoxy group on the central aromatic ring.

**Table 3. Scavenging Effects of 3,3″-Dihydroxyterphenyllin, 3-Hydroxyterphenyllin, and Candidusin B Isolated from the Ethyl Acetate Extracts of *A. candidus* (CCRC 31543) Broth Filtrate on DPPH Radicals**

| compound[a] | scavenging effect (%)[b] |
|---|---|
| control | 0.00 |
| 3,3″-di-OH terphyllin | 94.7 ± 0.5[ac] |
| 3-OH terphyllin | 96.0 ± 0.2[a] |
| candidusin B | 59.2 ± 4.9[c] |
| EtoAc extract | 84.4 ± 1.2[b] |
| BHA | 95.1 ± 1.2[a] |
| α-tocopherol | 94.7 ± 0.3[a] |

[a] The concentration of all compounds is 100 μg/mL. [b] Scavenging effect (%) = [1 - (absorbance of sample at 517 nm)/(absorbance of control at 517 nm)] × 100. [c] Each value is presented as mean ± SD ($n = 3$). Values within a column with different superscripts are significantly different ($p < 0.05$).

**Safety Evaluation.** The safety of 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B (10−200 μg/mL) was a concern and was therefore evaluated. The cell viability was greater than 95% when these three antioxidants were incubated with INT 407 cells at 37 °C for 30 min (data not shown). This means that these antioxidants showed no cytotoxicity toward human intestine cells at the tested concentrations. In addition, the mutagenicity of those three compounds toward *S. typhimurium* TA98 and TA 100 was investigated. Table 4 shows that no mutagenicity in *S. typhimurium* TA98 and TA100 was observed for 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B at 200 and 500 μg/mL per plate either with or without S9 mix. These results indicate that these three antioxidants did not induce base-pair substitution and frame-shift mutation toward *S. typhimurium* TA98 and TA100. Moreover,

**Table 4. Mutagenicity Ratio[a] of 3,3″-Dihydroxyterphenyllin, 3-Hydroxyterphenyllin, and Candidusin B Isolated from the Broth Filtrate Extracts of *A. candidus* (CCRC 31543) Produced by Fermentor[b] toward *S. typhimurium* TA98 and TA100 with and without S9 Mixture**

| sample (mg/plate) | 3,3″-di-OH terphyllin | | 3-OH terphyllin | | candidusin B | |
|---|---|---|---|---|---|---|
| | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 |
| | | | TA98 | | | |
| 0.2 | 1.84 | 1.15 | 1.72 | 0.90 | 1.88 | 1.06 |
| 0.5 | 1.89 | 0.92 | 1.91 | 1.10 | 1.94 | 1.15 |
| | | | TA100 | | | |
| 0.2 | 1.33 | 1.07 | 1.30 | 1.10 | 1.44 | 1.05 |
| 0.5 | 1.57 | 1.10 | 1.60 | 0.86 | 1.74 | 1.19 |

[a] Mutagenicity ratio = induced revertants per plate/spontaneous revertants per plate. The spontaneous revertants of TA98 and TA100 are 30 ± 8 and 188 ± 23, respectively. [b] The volume of fermentor is 100 L and working volume is 70 L.

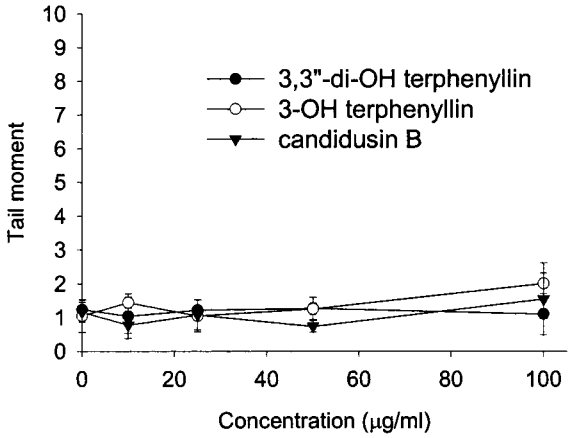



**Figure 3.** Genotoxicity of human intestine 407 (INT 407) cells treated with 3,3″-dihydroxyterphenyllin (-●-), 3-hydroxyterphenyllin (-○-), and candidusin B (-▼-) as measured by the single-cell comet assay. The DNA damage was expressed as tail moment, where tail moment = [tail length × tail DNA/(head DNA + tail DNA)] × 100. Results are mean ± SD for $n = 3$.

single-cell gel electrophoresis assay (comet assay) was employed to evaluate the DNA damage in cells directly caused by 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B. Moreover, no significant ($p > 0.05$) DNA damage was found when INT 407 cells were treated with these three antioxidants at concentrations ranging from 0 to 100 μg/mL (Figure 3).

It has been reported that 3,3″-di-OH terphenyllin and 3-OH terphenyllin could prolong the first cleavage cycle of sea urchin embryos (*4*) and 3-OH terphenyllin could inhibit plant growth (*29*). Candidusin B has been reported to inhibit DNA and RNA synthesis and provide an antibacterial activity against *Bacillus subtilis* (*18*). Apart from the latter studies, accomplished using microscope observation and feeding experiments, there have been no reports focusing on the toxicity of terphenyllin compounds. However, in this study the genotoxicity of these three antioxidants was evaluated and no cytotoxicity or mutagenicity was found through modern cell viability analysis, the Ames test, or the examination of direct DNA damage (comet assay). Therefore, the safety of 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B is still debatable. Because of this concern, further studies should be made to evaluate the effects of 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B on cell metabolism and animal physiology if these purified antioxidants are desired for use in food applications.

Results obtained in this study clearly demonstrate that the three isolated antioxidants, 3,3″-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B, from ethyl acetate extract of *A. candidus* filtrate, exhibited antioxidative activities equal to or higher than those of BHA and α-tocopherol with respect to the inhibition of linoleic acid peroxidation, prevention of lard oxidation, and scavenging effects on DPPH radicals. Safety evaluation of these antioxidants shows no cyto- or genotoxicity, and no mutagenicity by cell viability analysis, Ames test, or the single-cell comet assay. Further investigations to understand their antioxidant mechanisms more thoroughly and to examine their biomedical effects are underway in our laboratories.

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