Cytotoxicity and Nitric Oxide Production Inhibitory Activities of Compounds Isolated from the Plant Pathogenic Fungus Curvularia sp.

Virayu Suthiphasilp 1,†, Achara Raksat 1,†, Tharakorn Maneerat 1,2, Sarinya Hadsadee 3, Siriporn Jungsuttiwong 3, Stephen G. Pyne 4, Putarak Chomnunti 5, Wuttichai Jaidee 2, Rawiwan Charoen sup 2,6,* and Surat Laphookhieo 1,2,*†

1 Center of Chemical Innovation for Sustainability, School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand; virayu.suthiphasilp@gmail.com (V.S.); achara6299@gmail.com (A.R.); wisanu.man@mfu.ac.th (T.M.)
2 Medicinal Plants Innovation Center of Mae Fah Luang University, Chiang Rai 57100, Thailand; wuttichai.jai@mfu.ac.th
3 Center for Organic Electronic and Alternative Energy, Department of Chemistry and Center for Excellence for Innovation in Chemistry, Faculty of Science, Ubon Ratchathani University, Ubon Ratchathani 34190, Thailand; subaka29@gmail.com (S.H.); siripornj@ubu.ac.th (S.J.)
4 School of Chemistry and Molecular Bioscience, University of Wollongong, Wollongong, NSW 2522, Australia; spyne@uow.edu.au
5 Center of Excellence in Fungal Research, School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand; putarak.ch@mfu.ac.th
6 School of Integrative Medicine, Mae Fah Luang University, Chiang Rai 57100, Thailand
* Correspondence: rawiwan.cha@mfu.ac.th (R.C.); surat.lap@mfu.ac.th (S.L.)
† These authors have contributed equally to this work.

Abstract: Chemical investigation of the mycelia of the pathogenic fungus Curvularia sp. which was isolated from a leaf of Dactylctenium aegyptium (crowfoot grass), resulted in the isolation of a new compound, curvulariahawadride (5), along with five known compounds (1–4, and 6). Their structures were determined on the basis of spectroscopic data, including 1D and 2D NMR and HRESIMS. The absolute configuration of 5 was established from experimental and calculated electronic circular dichroism (ECD). Compounds 1, 3, and 5 showed nitric oxide (NO) production inhibitory activity with IC50 values of 53.7, 32.8, and 12.8 µM, respectively. Compounds 2 and 4 showed significant cytotoxicity against lung cancer A549, colorectal cancer SW480, and leukemic K562 cells with an IC50 ranging value of 11.73 to 17.59 µM.

Keywords: Curvularia sp.; Dactylctenium aegyptium; curvulariahawadride; cytotoxicity; nitric oxide production inhibitory activity

1. Introduction

Endophytic fungi are well-known as promising sources of structurally diverse and novel biologically active compounds [1–3]. Endophytic fungi have become one of the most important sources for drug discovery from nature because of the short period to scale up and the diversity of production of secondary metabolites [4,5]. Curvularia species are phytopathogenic fungi that are commonly isolated from soil or infected grasses and food plants, such as maize (Zea mays), oil palm (Elaeis guineensis), or rice (Oryza sativa) [6–8]. A major cause of leaf blight and leaf spot is infection by Bipolaris and Curvularia fungi and is considered one commonly associated with oil palm and corn leaf spots in Thailand [7,9]. In addition, several species of Curvularia have been reported for opportunistic infections in humans [10–12]. Previously, C. lunata was believed to be the most frequently reported human pathogenic species, while C. hawaiiensis (previously Bipolaris hawaiensis) was reported to infect the ear of a patient post-trauma [12,13]. Previously, cochlioquiones,
polyketides, terpenoids, alkaloids, quinones, and peptides have been isolated from the
 genus of Curvularia [14]. However, differences in fungal collections and fermentation
 conditions have yielded different compounds. Various types of compounds isolated from
 Curvularia species have shown impressive biological activities, including phytotoxic [15],
 antimicrobial [16–20], and cytotoxic [17,21,22] activities. Herein, we describe the isolation
 and structure elucidation of a new compound (5) along with five known compounds (1–4,
 and 6) from the ethyl acetate extract of mycelia from laboratory cultures of Curvularia sp.
 which was isolated from a leaf of D. aegyptium. In addition, the in vitro nitric oxide (NO)
 production inhibitory and cytotoxicities of the isolated compounds against three cancer
 cell lines, including lung cancer A549, colorectal cancer SW480, and leukemic K562 cells,
 and mammalian cells (RAW 264.7) are also reported.

2. Materials and Methods

2.1. General Experimental Procedures

The UV spectra were recorded with a Varian Cary 5000 UV-Vis-NIR spectrophotometer.
IR spectra were recorded on a Perkin Elmer FTS FT-IR spectrometer. The optical rotations
were measured with a Bellingham and Stanley APD440 polarimeter. Electronic circular
 dichroism spectra were recorded on a JASCO J-815 spectrometer. The NMR spectra were
recorded on a 400 MHz Bruker FT-NMR Ultra Shield. ESI-QIT-MS spectra were measured
on a Bruker-Hewlett-Packard 1100 Esquire-LC system mass spectrometer. Quick column
chromatography (QCC) and column chromatography (CC) were performed on silica
gel C60 (0–20 µm, Silicycle® Inc., Québec, QC, Canada) and silica gel G60 (60–200 µm,
Silicycle® Inc., Québec, QC, Canada), respectively. Sephadex LH-20 (25–100 µm, Merck,
Kenilworth, NJ, USA), where indicated, was also used for CC. Precoated TLC plates of
silica gel (60F254, Merck, Kenilworth, NJ, USA) were used for analytical purposes.

2.2. Fungal Material and Identification

A leaf spot symptom of crowfoot grass was collected from Hat Yai, Songkhla Province,
in July 2012. Fresh specimens were incubated for 1–2 days in a moist chamber to induce
sporulation. Fungi were isolated by a modified single spore suspension method [23]. Coni-
dia were taken from the fungal sporulation from leaf spot samples and placed in sterilized
water for spore suspension. The conidia were then transferred to water agar media and
left overnight to germinate, and germinated conidia were individually transferred to PDA.
The pure cultures of Curvularia sp. (MFLCC12-0192) were deposited in MFLUCC for future
study. Genomic DNA was extracted from fungal mycelium grown on PDA media using the
 Biospin Fungus Genomic DNA Extraction Kit (Bioflux®, Hangzhou, China), following the
instructions of the manufacturer. The DNA amplification was performed by polymerase
chain reaction (PCR). Primers ITS1 and ITS4 (Glass and Donaldson, 1995) were used to
amplify the 5.8S and ITS regions. The quality of PCR amplification was confirmed on 1% 
agarose gel electrophoresis stained with ethidium bromide. The amplified PCR fragments
were sent to the commercial sequencing provider (Shanghai Sangon Biological Engineering
Technology & Services Co., Shanghai, China).

2.3. Fermentation, Extraction, and Isolation

Fungal isolation Curvularia sp. (MFLCC12-0192) was grown on potato dextrose
agar (PDA) (Millipore, Merck, Kenilworth, NJ, USA) at 25 °C for 5 days. Five pieces
(0.5 × 0.5 cm²) of mycelial agar plugs were inoculated into Erlenmeyer flasks (2 L × 45),
each containing 400 mL of PDB media which were allowed to stand at room temperature
for 4 weeks. The culture was filtered to obtain the filtrate and mycelia. Wet mycelia were
extracted twice with 500 mL of MeOH. After the concentration of the MeOH solution to
100 mL, H₂O (100 mL) was added, and the mixture was extracted three times with EtOAc
(200 mL each). The ethyl acetate layer was concentrated under reduced pressure to obtain
a brown gum (2.4 g). The crude extract was subjected to quick column chromatography
(QCC, 10 × 20 cm) over silica gel (0–20 µm), eluting with a gradient of EtOAc-hexanes (0:5,
0.5:4.5, 1:4, 1.5:3.5, 2:3, 2.5:2.5, 3:1.5, 3.5:1.5, 4:1, 4.5:0.5, 0.5:4.5, and 5:0, v/v, 250 mL each) to afford three combined fractions (F1–F3). Fraction F2 (375.8 mg) was further purified by Sephadex LH-20 CC (25–100 µm, 13 × 50 cm) using 100% MeOH (2000 mL) and then followed by repeated silica gel CC (60–100 µm, 12 × 50 cm) using acetone/hexanes (2:8, v/v, 1500 mL) to afford compounds 1 (3.7 mg), 2 (14.5 mg), 3 (2.3 mg), 4 (15.6 mg), and 6 (4.8 mg). Compound 5 (2.1 mg) was obtained from fraction F3 (264.9 mg) by Sephadex LH-20 CC (25–100 µm, 13 × 30 cm) using 100% MeOH (2000 mL) and followed by silica gel CC (25–100 µm, 12 × 50 cm) using MeOH/CH₂Cl₂ (0.6:9.4, v/v, 1000 mL).

2.4. Bioassays

2.4.1. Nitric Oxide (NO) Production Inhibitory Assay

This assay was performed as previously described [24]. RAW 264.7 cells were seeded at 4 × 10⁴ cells/well in 96-well plates and incubated at 37 °C and 5% CO₂ overnight. Cells were incubated with 1 µg/mL LPS for 1 h and treated with various concentrations of tea extract, including 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL, for 24 h. After 24 h, 100 µL of Griess reagent was added to the samples for 10 min. Nitric oxide was measured at 570 nm with a Biochrom EZ Read 400 ELISA microplate reader (Biochrom Ltd., Cambridge, UK). Additionally, the data are presented as the IC₅₀, which was calculated with GraphPad Prism 6.0 software. Indomethacin was used as a positive control with an IC₅₀ value of 73.4 µM.

2.4.2. Cytotoxicity Assay in Mammalian Cells (RAW 264.7 Cells)

This assay was performed as previously described [25]. Cell viability was measured by the MTT assay. RAW 264.7 cells were seeded at 4 × 10⁴ cells/well in 96-well plates and incubated at 37 °C and 5% CO₂ overnight. Cells were treated with different concentrations of tea extracts, including 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL, for 24 h. After 24 h, cells were washed with PBS and incubated with 0.5 mM MTT reagent for 4 h. The detection of formazan at 570 nm was performed with a Biochrom EZ Read 400 ELISA microplate reader (Biochrom Ltd., Cambridge, UK). The data were calculated as IC₅₀ values with GraphPad Prism 6.0 software.

2.4.3. Cytotoxicity Assay against Lung Cancer A549, Colorectal Cancer SW480, and Leukemic K562 Cells

These assays were performed as previously described [25]. Lung cancer A549 and colorectal cancer SW480 cells were maintained with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Leukemic K562 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were cultured in 96-well plates at 37 °C in 5% CO₂, followed by treatment with the sample for 24 h. After the incubation period, 0.5 mg/mL MTT was added to the cells and left for 4 h. The formazan was dissolved in DMSO and measured at 570 nm using Biochrom EZ Read 400 ELISA microplate reader (Biochrom Ltd., Cambridge, UK).

2.5. Computational Methods

The electronic circular dichroism (ECD) calculations of compound 5 were carried out by using TD-DFT at the CAM-B3LYP functional with 6-311++G(d,p) basis. All structures were optimized by the DFT method at the B3LYP/6-31G (d,p) level of theory. Geometry optimization and TD-DFT computations were both performed with Continuum Model (PCM) solvation model with methanol. The rotary strengths of 100 excited states were calculated. All calculations were performed using Gaussian 09 program package [26]. Gaussian band shape with a bandwidth of 0.25 eV was used to simulate ECD spectra. The ECD curves were generated by the software SpecDis 1.64 (University of Wurzburg, Wurzburg, Germany).
3. Results and Discussion

3.1. Isolated Compounds from Curvularia sp.

The mycelia of Curvularia sp. were cultured on potato dextrose agar, and the mycelia were harvested, extracted, and subjected to repeated column chromatography to afford six compounds (Figure 1).

![Figure 1. Compounds isolated from Curvularia sp.](image)

3.2. Structural Characterization of a New Compound

Curvulariahawadride (5), [α]D25 +65 (c 0.1, MeOH), was isolated as a colorless viscous oil and its molecular formula was assigned as C_{18}H_{20}O_{6} based on the 13C NMR spectroscopic data and HRESIMS, which showed an ion peak at m/z 333.1339 [M + H]⁺ (calcd 333.1338). Its UV spectrum showed the characteristic absorption of a maleic anhydride chromophores at λ_max 252 nm [27,28]. The IR spectrum indicated the stretching bands of hydroxy (3298 cm⁻¹), anhydride carbonyl (1838 and 1763 cm⁻¹), and carboxylic acid carbonyl (1713 cm⁻¹) groups [27]. The 13C NMR (Table 1) and DEPT spectroscopic displayed 18 carbon resonances which were assigned to two methyls (δ_C 14.0 and 12.3), five methylenes (δ_C 34.9, 25.9, 24.3, 24.4, and 21.0), four carbonyls (δ_C 177.6, 169.8, 165.9, and 165.7), three sp³ methines (δ_C 46.3, 44.9, and 40.4), and four sp² carbons (δ_C 148.3, 146.9, 142.7, and 131.2). The 1D and 2D NMR data (Table 1) identified the presence of an ethyl group [δ_H/δ_C 1.98 (1H, ddd, J = 13.2, 7.2, 2.5 Hz, H-2″a) and 1.38 (1H, m, H-2″b)/25.9 and 0.93 (3H, t, J = 7.2 Hz, H-1″)/12.4], a propyl group [δ_H/δ_C 0.92 (3H, t, J = 7.6 Hz, H-1″)/14.0, 1.25 (2H, dd, J = 15.2, 7.6 Hz, H-2″)/21.0, and 1.89 (1H, m, H-3′a) and 1.53 (1H, m, H-3′b)/34.9], three methine protons [δ_H/δ_C 2.82 (1H, td, J = 10.5, 2.7 Hz, H-3)/40.4, 2.68 (1H, m, H-4)/46.3, and 3.49 (1H, d, J = 12.0 Hz, H-7)/44.8], two pairs of diastereotopic methylene protons [δ_H/δ_C 2.93 (1H, dd, J = 13.0, 7.3 Hz, H-9a) and 2.46 (1H, t, J = 13.0 Hz, H-9b)/24.4 and 2.69 (1H, m, H-8a) and 1.76 (1H, dd, J = 13.0, 12.6 Hz, H-8b)/24.7], and an olefinic proton [δ_H/δ_C 6.46 (1H, d, J = 11.7 Hz, H-5)/148.3]. Analysis of the 2D NMR spectra suggested that curvulariahawadride (5) had a polyketide nonadride skeleton.

Key HMBC correlations (Figure 2) of H-3 with C-1, C-5, C-10, C-2′, and C-2″ and H-9 with C-1, C-2, and C-11 suggested a five-membered anhydride nucleus on C-1 and C-2. COSY cross-peaks (Figure 2) between H-3/H-4, H-3/H-3′, H-4/H-5, and H-4/H-2″ confirmed the attachment of the ethyl and propyl substituents to C-4 and C-3, respectively. From the above information, it was apparent that compound 5 is a nonadride derivative, which has a unique nine-membered carbocyclic ring, previously found in heveadride (7) [27,28]. The correlations between H-3/H-2b′, H-4/H-1′, H-4/H-2′, H-5/H-3, H-5/H-1″, H-5/H-2b″, and H-7/H-9b in the NOESY experiment, suggested that H-3 and H-7 were located in the same side (α-orientation) but opposite to that of H-4 (β-orientation) (Figure S4). Moreover, the magnitude of the coupling constant (J = 10.5 Hz) of H-3 (δ_H 2.82) and H-4 (δ_H 2.68) suggested that these protons were in trans-orientation [27,28]. Finally, the absolute configuration of 5 was determined from the experimental and calculated electronic circular dichroism (ECD), as shown in Figure 3. Six putative structures of compound 5 (Figure S18) were optimized using B3LYP/6-31G(d,p) level in methanol.
(PCM) and the ECD spectrum for (3S,4R,7S)-5, (3R,4R,7S)-5, (3S,4S,7S)-5, (3S,4S,7R)-5, (3R,4S,7S)-5, and (3R,4S,7R)-5 were calculated at CAM-B3LYP/6-311++G(d,p) level in methanol (PCM) (Figure S19). Of these, the experimental ECD spectrum of 5 was similar to that of the computed ECD spectrum of (3S,4R,7S)-5 (Figure 3). Accordingly, the structure of compound 5 was proposed as (+)- (3S,4R,7S)-curvulariahawadride.

Table 1. $^1$H (400 MHz, CDCl$_3$) and $^{13}$C (100 MHz, CDCl$_3$) NMR Spectroscopic data of curvulariahawadride (5).

| Position | $\delta$C | $\delta$H [mult, J in Hz] | HMBC (1H $\rightarrow$ 13C) |
|----------|----------|------------------------|-------------------------|
| 1        | 142.7    |                        |                         |
| 2        | 146.9    |                        |                         |
| 3        | 40.4     | 2.82 (td, 10.5, 2.7)   | 1, 2, 4, 10, 3'          |
| 4        | 46.3     | 2.68 (m)               | 3                       |
| 5        | 148.3    | 6.46 (d, 11.7)         | 4, 6, 13, 2''           |
| 6        | 131.2    |                        |                         |
| 7        | 44.9     | 3.49 (d, 12.0)         | 5, 6, 8, 9, 12, 13      |
| 8        | 24.3     | 2.61 (m); 1.76 (dd, 13.0, 12.6) | 5, 6, 7, 9, 12 |
| 9        | 24.4     | 2.93 (dd, 13.0, 7.3); 2.46 (t, 13.0) | 1, 2, 7, 8, 11 |
| 10       | 165.9    |                        |                         |
| 11       | 165.7    |                        |                         |
| 12       | 177.6    |                        |                         |
| 13       | 169.8    |                        |                         |
| 1'       | 14.0     | 0.92 (t, 7.6)          | 2', 3'                  |
| 2'       | 21.0     | 1.25 (m)               | 3, 1', 3'               |
| 3'       | 34.9     | 1.89 (m); 1.53 (m)     | 1'                      |
| 1''      | 12.3     | 0.93 (t, 7.2)          | 4, 2''                  |
| 2''      | 25.9     | 1.98 (dd, 13.2, 7.2, 2.5); 1.38 (m) | 4, 5, 1'' |

Figure 2. COSY ($^1$H $\rightarrow$ $^1$H) and selected HMBC ($^1$H $\rightarrow$ $^{13}$C) correlations of 5.
Figure 3. ECD spectra of compound 5. Experimental and TDDFT calculated ECD spectra of compound 5 at CAM-B3LYP/6-311++G(d,p) level in methanol (PCM).

Five known compounds (1–4 and 6) were identified as cochlioquinone N (1) [29], cochlioquinone A (2) [30], stemphone (3) [31], anhydrocochlioquinone A (4) [32], and terpeatacin (6) [33] by comparison of their spectroscopic data with those in the literature. Compounds 1–4 were cochlioquinone derivatives containing a tetracyclic quinone skeleton in common. These compounds were isolated from Curvularia sp. for the first time. Previous reports showed that these compounds have been isolated from other genera of the Pleosporaceae family. Cochlioquinone N (1) had been isolated from Bipolaris sp. [27], while cochlioquinone A (2) had been found from Bipolaris and Cochliobolus genera, including B. sorokiniana [34], Bipolaris sp. [27], B. bicolor [35], Cochliobolus sp. [36], and C. miyabeanus [37]. Stemphone (3) has been reported from other genera of the Pleosporaceae family, including Drechslera sacchari [38], B. bicolor [35], and Stemphylium sarcinaeforme [29]. Anhydrocochlioquinone A (4) has also been isolated from B. oryzae [30], B. luttrellii [39], Cochliobolus sp. [36], and Veronaea sp. (Incertae sedis) [40]. Terpeatacin (6) is a bicyclic sesterterpene containing a 5-membered ring fusing with a 15-membered ring and was isolated for the first time in Curvularia sp. Terpeatacin (6) has been isolated from plant pathogenic fungi, including Arthrinium sp. (Apiosporaceae) [41], Neofusicoccum batangarum (Botryosphaeriaceae) [42], Simplicillium sp. (Cordycipitaceae) [43], Embellisia chlamydospora (Pleosporaceae) [44], Cochliobolus sp. (Pleosporaceae) [45], Fusarium culmorum (Nectriaceae) [46], and Bipolaris sorokiniana (Pleosporaceae) [47].

3.3. Cytotoxicity against the Three Cancer Cell Lines, including Lung Cancer A549, Colorectal Cancer SW480, and Leukemic K562 Cells and Mammalian Cells (RAW 264.7 Cells)

Compounds 1–6 were also evaluated for their cytotoxicities against the three cancer cell lines, lung cancer A549, colorectal cancer SW480, and leukemic K562 cells and mammalian cells, RAW 264.7 (Table 2). Compounds 1, 3, 5, and 6 were not cytotoxic at the concentration of 100 µg/mL, and their cell viability was in the range of 71.38 to 99.15%, whereas compounds 2 and 4 were cytotoxic with the cell viability in the range of 8.40 to 14.95%.
Table 2. Cell viability of compounds 1–6.

| Samples (100 µg/mL) | RAW 264.7 (Lung Cancer) | A549 (Lung Cancer) | SW480 (Colorectal Cancer) | K562 (Leukemic Cells) |
|---------------------|-------------------------|--------------------|---------------------------|-----------------------|
| 1                   | 97.14 ± 0.81            | 88.51 ± 1.19       | 82.60 ± 1.68              | 81.07 ± 3.80          |
| 2                   | 10.80 ± 0.09            | 13.37 ± 0.48       | 14.95 ± 0.57              | 12.54 ± 1.24          |
| 3                   | 71.38 ± 2.67            | 96.32 ± 3.70       | 75.59 ± 2.00              | 79.23 ± 1.86          |
| 4                   | 11.07 ± 0.43            | 8.40 ± 0.58        | 14.39 ± 0.40              | 10.56 ± 0.20          |
| 5                   | 82.59 ± 1.72            | 84.08 ± 3.01       | 97.87 ± 1.70              | 75.65 ± 1.19          |
| 6                   | 99.34 ± 2.51            | 92.03 ± 3.75       | 97.69 ± 1.04              | 99.15 ± 3.84          |
| 5% DMSO (control)   | 100.00 ± 2.47           | 100.00 ± 0.41      | 100.00 ± 2.49             | 100.00 ± 1.89         |

Table 3. Cytotoxicities of Cochlioquinone (2) and Anhydrocochlioquinone A (4).

| Sample | Concentration | Cell Viability (%) | RAW 264.7 (Lung Cancer) | A549 (Lung Cancer) | SW480 (Colorectal Cancer) | K562 (Leukemic Cells) |
|--------|---------------|--------------------|-------------------------|--------------------|---------------------------|-----------------------|
| 2      | 100           | 10.80 ± 0.09       | 13.37 ± 0.48            | 14.95 ± 0.57       | 12.54 ± 1.24              |                       |
|        | 50            | 11.67 ± 0.08       | 14.77 ± 2.54            | 15.64 ± 0.77       | 13.35 ± 0.89              |                       |
|        | 25            | 15.00 ± 0.29       | 17.92 ± 1.93            | 17.56 ± 0.70       | 21.01 ± 2.61              |                       |
|        | 12.5          | 16.61 ± 0.52       | 23.07 ± 1.84            | 23.41 ± 0.25       | 38.42 ± 3.18              |                       |
|        | 6.25          | 28.48 ± 1.98       | 73.11 ± 1.09            | 30.75 ± 0.40       | 70.95 ± 1.69              |                       |
|        | IC_{50} (µM)  | <11.73             | 14.34                   | <11.73             | 17.59                     |                       |
| 4      | 100           | 11.07 ± 0.43       | 8.40 ± 0.58             | 14.39 ± 0.40       | 10.56 ± 0.20              |                       |
|        | 50            | 12.79 ± 0.70       | 9.35 ± 0.52             | 14.71 ± 0.24       | 11.27 ± 0.40              |                       |
|        | 25            | 12.14 ± 0.42       | 10.16 ± 0.17            | 15.37 ± 0.42       | 12.88 ± 0.34              |                       |
|        | 12.5          | 12.45 ± 0.47       | 11.58 ± 1.05            | 15.59 ± 0.39       | 14.03 ± 2.31              |                       |
|        | 6.25          | 14.32 ± 0.73       | 12.39 ± 0.17            | 17.29 ± 0.33       | 19.48 ± 2.62              |                       |
|        | IC_{50} (µM)  | <12.14             | <12.14                  | <12.14             | <12.14                    |                       |

The previous report showed that anhydrocochlioquinone A (4) displayed weak cytotoxicity against lung cancer (A549) cell line with the inhibition ratio of 41% at 30 µg/mL [40]. In this study, anhydrocochlioquinone A (4) showed the cytotoxicity against lung cancer (A549) cell line with the IC_{50} value of <12.14 µM, which agrees with the previous report. It is interesting to note that compounds 1-4 shared the same core structure framework (cochlioquinone derivative); however, the cytotoxicities against the three cancer cell lines were different. Cochlioquinone A (2) was different from cochlioquinone N (1) (R_{11} = NH₂) at C-11 and stemphone (3) at C-2/3 (Δ²(3)). The contained amino group at C-11 of cochlioquinone N (1) and a double bond at C-2/3 (Δ²(3)) of stemphone (3) have much more effect...
on reducing the cytotoxicities against the three cancer cell lines. In the case of cochlioquinone A (2) and anhydrocochlioquinone A (4), cochlioquinone A (2) contained a hydroxy group at C-12, while anhydrocochlioquinone A (4) was a dehydration form at C-12/13 (\(\Delta^{12(13)}\)). This difference had no significant effects on cytotoxicity.

3.4. Nitric Oxide Production Inhibitory Activity of Isolated Compounds 1–6

Compounds 1–6 were screened for their NO production inhibitory activity. At the concentration of 100 \(\mu\)g/mL, compounds 1, 3, 5, and 6 showed NO production inhibitory with the percentage inhibition ranging from 41.03 to 74.01% and the IC50 values ranging from 12.9–53.7 \(\mu\)M (Table 4). Cochlioquinone A (2) and anhydrocochlioquinone A (4) were not tested due to these compounds leading to cell death. Curvulariahawadride (5) showed the best NO production inhibition activity with an IC50 value of 12.9 \(\mu\)M, which was better than the positive control, indomethacin (IC50 value of 73.4 \(\mu\)M). Cochlioquinone N (1) and stemphone (3) also showed NO production inhibition activity better than the positive control with IC50 values of 53.7 and 32.8, respectively. The other compounds (2, 4, and 6) were inactive at the concentration of 100 \(\mu\)g/mL. Notably, this is the first publication on the NO production inhibitory activity of these compounds.

Table 4. Nitric oxide production inhibition activity of compounds 1–6.

| Samples | % of NO Inhibition at 100 \(\mu\)g/mL | IC50 (\(\mu\)M) |
|---------|--------------------------------------|---------------|
| 1       | 51.30 ± 1.65                         | 53.7           |
| 2       | not tested *                         | not tested *  |
| 3       | 73.41 ± 0.44                         | 32.8           |
| 4       | not tested *                         | not tested *  |
| 5       | 74.01 ± 1.14                         | 12.8           |
| 6       | 41.03 ± 3.36                         | inactive       |
| Indomethacin | 77.45 ± 0.28               | 73.4           |

* Not tested due to compound leads to cell death.

4. Conclusions

The chemical investigation of plant pathogenic fungus Curvularia sp. led to the isolation and identification of a new nonadride derivative, curvulariahawadride (5), together with five known compounds. Cochlioquinones were found as major compounds from this study. All compounds were evaluated for their cytotoxicities against the three cancer cell lines, lung cancer A549, colorectal cancer SW480, and leukemic K562 cells, and NO production inhibitory activity. Only two compounds (2 and 4) showed cytotoxicities against the three cancer cell lines. In the case of NO production inhibitory activity, compound 5 showed the best NO production inhibitory activity, which better than the positive control.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7060408/s1. The \(^1\)H and \(^{13}\)C NMR spectroscopic data of all known compounds. Figure S1: \(^1\)H NMR (400 MHz, CDCl3) of curvulariahawadride (5), Figure S2: \(^{13}\)C NMR (100 MHz, CDCl3) of curvulariahawadride (5), Figure S3: COSY (400 MHz, CDCl3) of curvulariahawadride (5), Figure S4: NOESY (400 MHz, CDCl3) of curvulariahawadride (5), Figure S5: HSQC (400 MHz, 100 MHz, CDCl3) of curvulariahawadride (5), Figure S6: HMBC (400 MHz, 100 MHz, CDCl3) of curvulariahawadride (5), Figure S7: HRESITOFMS spectrum of curvulariahawadride (5), Figure S8: \(^1\)H NMR (400 MHz, CDCl3) of compound 1, Figure S9: \(^{13}\)C NMR (100 MHz, CDCl3) of compound 1, Figure S10: \(^1\)H NMR (400 MHz, acetone-\(d_6\)) of compound 2, Figure S11: \(^{13}\)C NMR (100 MHz, acetone-\(d_6\)) of compound 2, Figure S12: \(^1\)H NMR (400 MHz, acetone-\(d_6\)) of compound 3, Figure S13: \(^{13}\)C NMR (100 MHz, acetone-\(d_6\)) of compound 3, Figure S14: \(^1\)H NMR (400 MHz, CDCl3) of compound 4, Figure S15: \(^{13}\)C NMR (100 MHz, CDCl3) of compound 4, Figure S16: \(^1\)H NMR (400 MHz, CDCl3) of compound
6, Figure S17: $^{13}$C NMR (100 MHz, CDCl$_3$) of compound 6, Figure S18. Optimized structures of compounds 5a-5f using B3LYP/6-31G(d,p) level in methanol (PCM). Figure S19. ECD spectra of compounds 5a-5f compare with experimental ECD compound 5.

**Author Contributions:** Conceptualization, S.L. and R.C.; methodology, A.R., VS., S.H., S.J., W.J., R.C., and P.C.; formal analysis, A.R., VS., TM., S.H., S.J., S.G.P., R.C., and S.L.; investigation, A.R., VS., and R.C.; resources, P.C., TM., R.C., and S.L.; data curation, A.R., VS., S.H., W.J., R.C., and S.J.; writing—original draft preparation, A.R., VS., TM., W.J., R.C., S.G.P., and S.L.; writing—review and editing, A.R., VS., S.G.P., R.C., and S.L.; funding acquisition, S.L. and R.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the Thailand Research Fund and Mae Fah Luang University through the Advanced Research Scholar (BRG5980012) and the Thailand Science Research and Innovation (DBG6280007). The Postdoctoral Fellowship from Mae Fah Luang University to VS. was also acknowledged.

**Data Availability Statement:** Data included in article/Supplementary Materials/referenced in the article.

**Acknowledgments:** We would like to thank Mae Fah Luang University, University of Wollongong, and University of British Columbia for laboratory facilities. This research was also supported by the Postdoctoral Fellowship (VS.) from Mae Fah Luang University.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Gao, H.; Li, G.; Lou, H.-X. Structural diversity and biological activities of novel secondary metabolites from endophytes. *Molecules* 2018, 23, 646. [CrossRef] [PubMed]
2. Uzma, F.; Mohan, C.D.; Siddaiah, C.N.; Chowdappa, S. Endophytic Fungi: Promising Source of Novel Bioactive Compounds. *In Fungal Biology*; Gupta, V.K., Tuohy, M.G., Eds.; Springer Nature Switzerland AG: Cham, Switzerland, 2019; pp. 243–265. [CrossRef]
3. Daley, S.-K.; Cordell, G.A. Biologically significant and recently isolated alkaloids from endophytic fungi. *J. Nat. Prod.* 2021, 84, 871–893. [CrossRef]
4. Aly, A.H.; Debbab, A.; Proksch, P. Fifty years of drug discovery from fungi. *Fungal Divers.* 2011, 50, 3–19. [CrossRef]
5. Rajamanikyam, M.; Vadlapudi, V.; Amarchy, R.; Upadhyayula, S.M. Endophytic fungi as novel resources of natural therapeutics. *Braz. Arch. Biol. Technol.* 2017, 60, 60. [CrossRef]
6. Sánchez-Márquez, S.; Bills, G.F.; Zabalgoa-Maza, I. Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. *Fungal Divers.* 2008, 3, 1–7.
7. Sunpapao, A.; Kittimorakul, J.; Pornsuriya, C. Disease note: Identification of *Curvularia oryzae* as cause of leaf spot disease on oil palm seedlings in nurseries of Thailand. *Phytoparasitica* 2014, 42, 529–533. [CrossRef]
8. Kusai, N.A.; Azmi, M.M.Z.; Zulkifly, S.; Yusof, M.T.; Zainudin, N.A.I.M. Morphological and molecular characterization of *Curvularia* and related species associated with leaf spot disease of rice in Peninsular Malaysia. *Rendiconti Lincei. Scienze Fisiche e Naturali* 2016, 27, 205–214. [CrossRef]
9. Marin-Felix, Y.; Senwanna, C.; Cheewangkoon, R.; Crous, P.W. New species and records of *Bipolaris* and *Curvularia* from Thailand. *Mycosphere* 2017, 8, 556–1574. [CrossRef]
10. Kamalam, A.; Ajithdass, K.; Sentamilselvi, G.; Thambiah, A.S. Paronychia and black discoloration of a thumb nail caused by *Curvularia lunata*. *Mycopathologia* 1992, 118, 83–84. [CrossRef]
11. Ebright, J.R.; Chandrasekar, P.H.; Marks, S.; Fairfax, M.R.; Aneziokoro, A.; McGinnis, M.R. Invasive sinusitis and cerebritis due to *Curvularia clavata* in an immunocompetent adult. *Clin. Infect. Dis.* 1999, 28, 687–689. [CrossRef]
12. Gunasekaran, S.; Tupaki-Sreepurna, A.; Thanneru, V.; Sekar, U.; Shanthi, M.; Kindo, A.J. A rare case of *Curvularia ha-wa-wiensis* in the ear following trauma. *J. Med. Sci. Clin. Res.* 2017, 5, 28154–28158. [CrossRef]
13. McAleer, R.; Kroenert, D.B.; Elder, J.L.; Froudist, J.H. Allergic bronchopulmonary disease caused by *Curvularia pallescens*. *Tetrahedron* 1995, 51, 4947–4952. [CrossRef]
14. Han, W.B.; Lu, Y.H.; Zhang, A.H.; Zhang, G.F.; Mei, Y.N.; Jiang, N.; Lei, X.; Song, Y.C.; Ng, S.W.; Tan, R.X. Curvulamine, a new antibacterial alkaloid incorporating two undescribed units from a *Curvularia* Species. *Org. Lett.* 2014, 16, 5366–5369. [CrossRef] [PubMed]
17. Zhang, M.-Q.; Xu, K.-X.; Xue, Y.; Cao, F.; Yang, L.-J.; Hou, X.-M.; Wang, C.-Y.; Shao, C.-L. Sordarin diterpene glycosides with

18. Campos, F.F.; Ramos, J.P.; De Oliveira, D.M.; Alves, T.M.A.; De Souza-Fagundes, E.M.; Zani, C.L.; Sampaio, F.C.; Converti, A.;

19. Greve, H.; Schupp, P.J.; Eguereva, E.; Kehraus, S.; König, G.M. Ten-membered lactones from the marine-derived fungus

20. Kaaniche, F.; Hamed, A.; Abdel-Razek, A.S.; Wibberg, D.; Abdissa, N.; El Euch, I.Z.; Allouche, N.; Melloulı, L.; Shaaban, M.;

21. Greve, H.; Schupp, P.J.; Eguereva, E.; Kehraus, S.; Kelter, G.; Maier, A.; König, G.M.; Fiebig, H.-H. Apralactone A and a new

22. Greve, H.; Schupp, P.J.; Eguereva, E.; Kehraus, S.; Kelter, G.; Maier, A.; Koenig, G.M.; Fiebig, H.-H. Apralactone A and a new

23. Chomnunti, P.; Schoch, C.L.; Aiguirre-Hudson, B.; Ko-Ko, T.W.; Hongsanan, S.; Jones, E.B.G.; Kodsube, R.; Phookamsak, R.;

24. Joo, T.; Sowndhararajan, K.; Hong, S.; Lee, J.; Park, S.-Y.; Kim, S.; Jhoo, J.-W. Inhibition of nitric oxide production in LPS-stimulated

25. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Scalmani, G.; Barone, V.; Mennucci, B.;

26. Crane, R.I.; Hedden, P.; MacMillan, J.; Turner, W.B. Fungal products. part IV. The structure of heveadride, a new nonadride from

27. Jhung, H.J.; Lee, H.B.; Lim, C.-H.; Kim, C.-J.; Kwon, H.J. Cochlioquinone A1, a new anti-angiogenic agent from

28. Berestetskiy, A.O.; Dalinova, A.A.; Dubovik, V.R.; Grigoryeva, E.N.; Kochura, D.M.; Senderskiy, I.V.; Smirnov, S.N.; Stepanycheva,

29. Miyagawa, H.; Nagai, S.; Tsurushima, T.; Sato, M.; Ueno, T.; Fukami, H. Phytotoxins produced by the plant pathogenic fungus

30. Jung, H.J.; Lee, H.B.; Lim, C.-H.; Kim, C.-J.; Kwon, H.J. Cochlioquinone A1, a new anti-angiogenic agent from Bipolaris zeicola.

31. Huber, C.; Court, W.; Devlin, J.; Edwards, O.; Scott, P. Stemphone: A new type of natural quinone. 

32. Phuwwapraisirisan, P.; Sawangk, P.; Pippong, T. Anhydrocicoquinone A, a new antitumor compound from Bipolaris oryzae. 

33. Oka, M.; Iimura, S.; Tenmyo, O.; Sawada, Y.; Sugawara, M.; Ohkusa, N.; Yamamoto, H.; Kawanoko, K.; Hu, S.-L.; Fukagawa, Y.;

34. Berestetskiy, A.O.; Dalinova, A.A.; Dubovik, V.R.; Grigoryeva, E.N.; Kochura, D.M.; Senderskiy, I.V.; Smirnov, S.N.; Stepanycheva,

35. Miyagawa, H.; Nagai, S.; Tsurushima, T.; Sato, M.; Ueno, T.; Fukami, H. Phytotoxins produced by the plant pathogenic fungus

36. Campos, F.F.; Ramos, J.P.; De Oliveira, D.M.; Alves, T.M.A.; De Souza-Fagundes, E.M.; Zani, C.L.; Sampaio, F.C.; Converti, A.;

37. Carruthers, J.R.; Cerrini, S.; Fedeli, W.; Casinovi, C.G.; Galeffi, C.; Vaccaro, A.M.T.; Scala, A. Structures of cochlioquinones A

38. Ogawara, H.; Higashi, K.; Machida, T.; Takashima, J.; Chiba, N.; Mikawa, T. Inhibitors of diacyl glycerol kinase from Drechslera

39. Qi, Q.-Y.; Huang, L.; He, L.-W.; Han, J.-J.; Chen, Q.; Cai, L.; Liu, H.-W. Cochlioquinone derivatives with apoptosis-inducing effects

40. Zhou, L.; Zheng, X.; Wan, C.-P.; Yu, Z.-F.; Zhang, K.-Q.; Li, G.-H. Chemical constituents of the fungus Veronaea sp. Chem. Nat. 

41. Ye, B.; Ding, W.; Wang, P.-M.; Xu, J. Two new sesterterpenes from marine-derived fungus Arthrinium sp. Chem. Nat. Compd. 2019,

10 of 11
42. Masi, M.; Aloj, F.; Nocera, P.; Cacciola, S.O.; Surico, G.; Evidente, A. Phytotoxic metabolites isolated from Neofusicoccum batangarum, the causal agent of the scabby canker of cactus pear (Opuntia ficus-indica L.). Toxins 2020, 12, 126. [CrossRef]
43. Saetang, P.; Rukachaisirikul, V.; Phongpaichit, S.; Preedanon, S.; Sakayaroj, J.; Borwornpinyo, S.; Seemakhan, S.; Muanprasat, C. Depsidones and an α-pyrene derivative from Simplicillium sp. PSU-H41, an endophytic fungus from Hevea brasiliensis leaf. Phytochemistry 2017, 143, 115–123. [CrossRef] [PubMed]
44. Jung, H.J.; Lee, H.B.; Kim, C.J.; Rho, J.-R.; Shin, J.; Kwon, H.J. Anti-angiogenic activity of terpestacin, a bicyclo sesterterpene from Embellisia chlamydospora. J. Antibiot. 2003, 56, 492–496. [CrossRef] [PubMed]
45. Zhang, G.F.; Guo, Z.K.; Wang, W.; Cui, J.T.; Tan, R.X.; Ge, H.M. Neuraminidase inhibitory terpenes from endophytic Cochliobolus sp. J. Asian Nat. Prod. Res. 2011, 13, 761–764. [CrossRef] [PubMed]
46. Schlegel, B.; Schmidtke, M.; Dörfelt, H.; Kleinwächter, P.; Gräfe, U. (−)-Terpestacin and L-tenuazonic acid, inducers of pigment and aerial mycelium formation by Fusarium culmorum JP 15. J. Basic Microbiol. 2001, 41, 179–183. [CrossRef]
47. Nihashi, Y.; Lim, C.-H.; Tanaka, C.; Miyagawa, H.; Ueno, T. Phytotoxic sesterterpene, 11-epiterpestacin, from Bipolaris sorokiniana NSDR-011. Biosci. Biotechnol. Biochem. 2002, 66, 685–688. [CrossRef] [PubMed]