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Thesis for Master Degree

New Influenza A (H1N1) Neuraminidase Inhibitory Curcuminoids from *Curcuma longa* L.

Chosun University Graduate School

Department of Pharmacy

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약학과

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List of Abbreviations

$[\alpha]_{TD}$: specific rotation

CD: circular dichroism

COSY: H-H correlation spectroscopy

DMSO: dimethyl sulfoxide

EC_{50} mean (50%) value of effective concentration

HMBC: heteronuclear multiple bond correlation

HMQC: heteronuclear multiple quantum coherence

HPLC: high performance liquid chromatography

HR-EI-MS: high resolution electro impact mass spectroscopy

IC_{50}: mean (50%) value of cytotoxic concentration

IR: infrared absorption

m/z: mass to charge ratio

NA: neuraminidase

NMR: nuclear magnetic resonance

NOESY: nuclear overhauser effect spectroscopy

Ppm: parts per million

RP: reverse phase

SI: selective index

UV: ultraviolet absorption

WT: wild type

(국문 초록)

울금 (*Curcuma longa* L.)로부터 신종 인플루엔자 A (H1N1) 뉴라미니다제 저해제인 커큐미노이드

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고 병원성 인플루엔자 A 바이러스인 신종 H1N1 인플루엔자의 출현으로 전 세계 사람들의 건강에 지속적으로 심각한 위협을 주고 있다.

본 연구자는 신종 H1N1 인플루엔자의 방어대책에 시급성을 감안하여 항인플루엔자 효과가 있는 물질들을 탐색하는 과정에서 메탄올로 추출한 *curcuma longa L.*로부터 신종플루 (H1N1)에 대하여 저해활성을 갖는 신규화합물 3 개와 기지화합물인 10 개의 curcuminoid 물질들을 분리 하였다. 분리한 모든 curcuminoid 물질들은 2 개의 유행성 바이러스 종인 H1N1 과 H9N2 에서 뉴라미니다제 저해효과가 있는 걸로 나타났으며 저해형식은 뉴라미니다제에 대하여 non-competitive 방법으로 저해하였다. IC_{50} 값의 범위는 H1N1 과 H9N2 바이러스 각각에 대하여 6.18~40.17μg/mL 과 3.77~31.82μg/mL 로서 좋은 저해효과가 나타났다.

최근 조류독감 치료제로 사용되고 있는 타미플루에 내성을 보이는 조류독감 바이러스의 출현은 인류의 보건에 심각한 위협을 초래하고 있다. 본 연구자는 타미플루의 내성을 보이는 신종플루 뉴라미니다제 효소를 인위적으로 클로닝하여 만들고 이들 타미플루에 내성을 갖는 뉴라미니다제에 대하여 화합물의 활성을

검색한 결과 curcuminoid 물질들은 타미플루 내성 신종플루 뉴라미니다제의 저해활성이 있음을 확인하였다.

타미플루 내성 뉴라미나다제에서 타미플루를 사용하는 경우 타미플루의 저해활성이 현저히 약화되었다. 그러나, 화합물 9 번과 타미플루를 병용하여 처리하였을 경우 타미플루 내성 뉴라미니다제에 대한 타미플루의 저해활성이 유지됨을 확인함으로서 *Curcuma longa* 에서 분리한 curcuminoid 물질들이 신종플루 및 타미플루 내성 신종플루의 뉴라미니다제 저해제로 이용될 수 있음을 확인하였다.

ABSTRACT

New Influenza A (H1N1) Neuraminidase Inhibitory Curcuminoids from *Curcuma longa* L.

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The emergence of drug-resistant influenza viruses and the threat of pandemics highlight the need for new and effective antiviral agents. As part of an ongoing anti-influenza screening program of natural products, three new (**1**–**3**) and ten known (**4**−**13**) curcuminoids were isolated from a methanol extract of *Curcuma longa* L. All compounds had strong inhibitory effects on the neuraminidases from two influenza viral strains, H1N1 and H9N2, as noncompetitive inhibitors with IC_{50} values ranging from 6.18 to 40.17μg/mL and 3.77 to 31.82 μg/mL, respectively. Furthermore, they exhibited significant inhibitory activity against the neuraminidases from novel influenza H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y mutant) expressed in 293T cells. In addition, the detailed relationships of structure and activity by the compounds were investigated. These results suggest that the curcuminoids from *C. longa* may be potential supplemental

molecules in the battle against influenza A (H1N1).

1. Introduction

1.1 Influenza A (H1N1) virus

Influenza is an infectious disease caused by RNA viruses, which afflicts large parts of the population in annual epidemic outbreaks. It often causes severe, sometimes even lethal, effects. Since the virus is highly contagious and transmitted by aerosol, it spreads rapidly. There is always the threat of particularly virulent strains, which lead to more and more cases of disease and death [1].

These viruses belong to the Orthomyxoviridae family and are classified in three types A, B, and C, among them the type A is clinically the most important[2]. Type A viruses account for all of the human pandemics of the last century: the 1918 H1N1 "Spanish," the 1957 H2N2 "Asian," and the 1968 H3N2 "Hong Kong" influenza viruses[3]. The 1918 influenza pandemic remains the most devastating single pandemic of any infectious disease in recorded history. The virus pandemic spread globally, infecting 25 to 30% of the world's population and killing at least 20 to 50 million worldwide, including more than half a million people in the United States[4]. Since April 2009, a novel swine-origin influenza A (H1N1) virus (S-OIA) was identified from the patients in Mexico, USA, Canada or elsewhere. On June 11, 2009, the World Health Organization (WHO) raised the worldwide pandemic alert level to Phase 6 in response to the ongoing global spread of the novel influenza A (H1N1) virus[5-6]. To prevent high mortality during international spread, a strategic approach is needed. Vaccination is the primary tool to control these viral infections, but rapid use of antiviral drugs can help eliminate epidemics and ameliorate the symptoms and burden of infected people. Thus, together with vaccination, antiviral drugs likely will play an important role in reducing the severity and

spread of infection during the pandemic [7].

1.2 Neuraminidase inhibitors

Influenza virus can be classified by the antigenic properties of two surface glycoproteins, namely hemaglutinine and neuraminidase. Sixteen subtypes have currently been defined for the hemaglutinine protein (H1-H16) and nine for the neuraminidase protein (N1-N9). The hemaglutinine antigen binds to the sialic acid receptor on the cell surface, which mediates the virus entry. The neuraminidase (NA) is one of the two major surface proteins in both type A and type B influenza viruses. NA is an attractive target for antiviral strategy because of its essential role in the pathogenicity of many respiratory viruses. Neuraminidase cleaves the specific linkage of the sialic acid receptor, resulting in the release of the newly formed virions from the infected cells. The genetic stability of the NA enzymatic active center among influenza viruses makes it a promising target for the development of antiviral drugs aimed at protecting humans against all influenza viruses. Additionally, the neuraminidase may function to facilitate the early process of influenza virus infection of lung epithelial cells. Hence, neuraminidase has been an attractive target for the development of novel anti-influenza drugs [8].

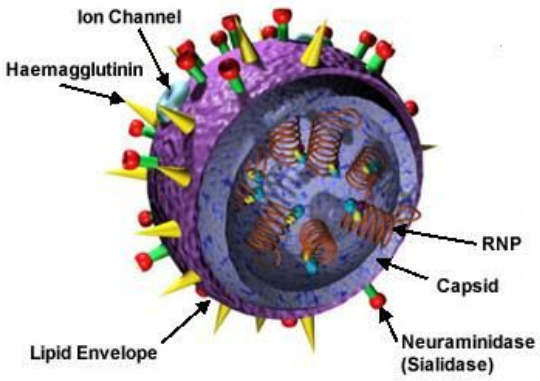

Figure 1. Structural diagram of the Influenza virus

The first antiinfluenza drugs were adamantine and its analogue rimantadine which block the hydrogen ion channel activity of the M2 protein of influenza A virus, inhibiting viral replication by blocking virus entry into cells [9]. However, they are only active against type A influenza viruses and the clinical use of amantadine has been limited since many side effects on central nervous system. Because of a broader antiviral spectrum, better tolerance, and less potential for emergence of resistance than was shown with the M2 inhibitors, the neuraminidase inhibitors represent an important advance in the treatment of influenza[10].

Two drugs that have been launched in the last years zanamivir (2,4-dideoxy-2,3-didehydro-4-guanidinosialic acid) and the orally active prodrug oseltamivir (ethyl 4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate; Tamiflu) present the latest class of antiinfluenza drugs, the neuraminidase (NA) inhibitors. Zanamivir is administered by oral inhalation, and oseltamivir is a prodrug that is converted to the active form upon oral administration. The world is currently almost completely dependent on these two licensed drugs for the treatment or prevention of seasonal (influenza A and B viruses) and pandemic influenza (influenza A viruses).

Unlike amantadine and rimantadine that target the M2 protein of influenza A viruses, these drugs inhibit replication of both influenza A and B viruses. On hydrolysis by hepatic esterases, the active carboxylate, is exposed to interact with three arginine residues (Arg118, Arg292, and Arg371) in the active site of NA [11]. Recently, however, significant levels of oseltamivir-resistant influenza A (H1) seasonal influenza viruses have also been encountered, which has been associated with a single amino acid change in the viral neuraminidase (H274Y) [12]. Due to the high prevalence of H1N1 2009 seasonal influenza isolates which display H274Y associated oseltamivir-resistance, antiviral drug resistance for influenza therapies remains a concern. Furthermore, the emergence of novel H1N1 raises the potential that additional

reassortments can occur, resulting in drug resistant virus[13]. Therefore, additional antiviral approaches are urgently needed and it is desirable to develop the novel antiviral medicines which overcome the drug resistance in some patients who received oseltamivir treatment [14].

1.3 Curcuma longa

Curcuma longa (Zingiberaceae), turmeric, is a plant belonging to the ginger family found in south and southeast tropical Asia [15]. It is widely used as food and medicine. Tumeric is a perennial rhizomatous herb, having stout, fleshy, main rhizome nearly ovoid, about 3 cm in diameter and 4 cm long. Lateral rhizomes, slightly bent, 1cm in diameter and 2-6 cm long. Rhizome flesh presents orange yellow color. Leaves emerge directly from underground stem with overlapping petioles, lanceolate, uniformly green, up to 30-50 cm long and 7-25 cm wide; apex acute and caudate with tapering base. Petiole and sheet are sparsely to densely pubescent. Blades are thin ellipse-shaped or elongate lance-shaped. Inflorescence (spike) is cylindrical, about 10-15 cm long and 5-7 cm in diameter, appearing with the leaves and developing in their centre. It consists of imbricate bracts, 5-6 cm long, pouch-like curved shape, white or white with light green color at upper half.

A great number of compounds have been identified as constituents in turmeric especially in rhizomes. Its rhizome contains phenolic compounds, terpenoids[16-17], polysaccharide, and fatty acids[18-19]. Phenolic compounds named curcuminoids are yellow pigments in the rhizomes. Total curcuminoids has been found 3-5% of rhizome[17]. Terpenoids are constituents in volatile oil of turmeric[15]. In rhizomes, volatile oil has been found 2-7%[17]. Major compounds vary depending on forms of material, locations of cultivation and analysis methods. .

There are several reports on a variety of pharmacological activities of curcuminoids including cardiovascular protection, anti-tumor, antioxidant, anti-inflammatory, anti-alzheimer,

anti-hepatotoxic and anti-virus [20-26].

During the course of an anti-influenza screening program from natural products [27-28], a methanol extract of *C. longa* was found to exhibit potential NA inhibitory properties. Although anti-viral activity of curcumin was reported[12], detailed structure and activity relationships by compounds from *C. longa* were not reported. Furthermore, there are no reports of the anti-viral activities of *C. longa* on swine flu (H1N1) and tamiflu-resistant swine flu. Thus, this prompted us to identify the active principles with NA inhibitory activity of *C. longa* by bioactivity-guided fractionation. This research describes the isolation and structural elucidation of three new (**1**–**3**) and ten known (**4**−**13**) curcuminoids from a methanol extract of *Curcuma longa* L. Additionally, anti-viral activity of curcuminoids was determined on NAs from two influenza viral strains, H1N1 and H9N2, and also on NAs from both novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells.

2. Materials and Methods

2.1 Materials

2.1.1 General experimental procedures

Optical rotations were determined on a Rudolph Autopol IV polarimeter using a 100 mm glass microcell. UV spectra were recorded in MeOH on a JASCO V-550 UV/VIS spectrometer. IR spectra (KBr) were recorded on a Nicolet 6700 FT-IR (Thermo electron Corp.). NMR spectra were obtained on a Varian Inova 500 MHz spectrometer with TMS as the internal standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The EIMS and HREIMS data were performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63–200 μm particle size), RP-18 (Merck, 40–63 μm particle size), and Sephadex LH-20 were used for column chromatography. TLC was carried out with silica gel 60 F254 and RP-18 F254 plates. HPLC was carried out using a Gilson system with a UV detector and an Optima Pak C18 column (10 × 250 mm, 10 μm particle size, RS Tech, Korea). All solvents used for extraction and isolation were of analytical grade.

2.1.2 Plant material

The dried roots of *C. longa* was purchased from Kangwon Herbal Medicine Company (Kangwondo, Republic of Korea), in June 2009 and identified by one of us (Prof. W.K.O.). A voucher specimen (CU2009–06) has been deposited at the Herbarium of Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju 501–759, Republic of Korea.

2.2 Methods

2.2.1 Extraction and isolation

The dried rhizomes of *C. longa* (3 kg) were extracted with methanol (15 L × 3 times) at room temperature for a week. The combined methanol extract was then concentrated to yield a dry residue (380 g). This crude extract was suspended in water (2.5 L) and partitioned successively with n-hexane (3 × 2 L), EtOAC (3 × 2 L), and BuOH (3 × 2 L). The EtOAC and BuOH soluble fractions, which showed strong influenza NA inhibitory activity, were combined (130 g) and chromatographed over a silica gel column (10 × 30 cm; 63–200 μm particle size) eluting with gradient solvent $CHCl_3$/acetone (19:1, 18:2…1:19, each 2.5 L), to yield six fractions (F1: 17.6 g; F2: 8.5 g; F3: 5.6 g; F4: 9.8 g; F5: 10.5 g; F6: 12.6 g; F7: 22.4 mg) based on the TLC profile. The crystallization of fraction F3 from n-hexane/EtOAc afforded compound **13** (curcumin, 4.2 g). Fraction F4 was further applied to a normal-phase silica gel column (5 × 40 cm; 40–63 □m particle size) with a stepwise gradient of $CHCl_3$/MeCN (9:1, 8:2…1:9, each 2L) to afford five subfractions (F4.1–F4.5). Crystallization of subfractions F4.2 and F4.3 from chloroform gave compounds **4** (demethoxycurcumin, 1.3 g) and **5** (bisdemethoxycurcumin, 2.5 g), respectively. Fraction F4.4 (90 mg) was separated by HPLC [Optima Pak C18 column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeCN in H2O containing 0.1% formic acid (0–40 min: 45% MeCN, 40–45 min: 100% MeCN, 45–55 min: 100% MeCN); flow rate 2 mL/min; UV detection at 205 and 254 nm] to give compounds **8** (t_R = 30.0 min, 5.5 mg) and **10** (t_R = 34.0 min, 14.0 mg).

Fraction F5 was chromatographed over a Sephadex LH-20 column (7 × 40 cm) using MeOH as the eluting solvent to afford three subfractions (F5.1–F5.3). Subfraction F5.2 (3.1 g) was further chromatographed over a silica gel column (5 × 40 cm; 40–63 μm particle size) with a gradient solvent of $CHCl_3$/ MeCN (9:1, 8:2…1:9, each 2.5 L) to yield five fractions (F5.2.1–

F5.5.5). Subfraction F5.2.2 (150 mg) was purified by HPLC (0–45 min: 60% MeCN, 45–50 min: 100% MeCN) to yield compounds **9** (t_R = 35.0 min, 10.5 mg) and **11** (t_R = 38.5 min, 13.0 mg). Further separation of F5.2.3 (110 mg) by HPLC (0–65 min: 57% MeCN, 65–70 min: 100% MeCN) resulted in the isolation of compounds **6** (t_R = 42.0 min, 10.5 mg), **7** (t_R = 58.0 min, 7.5 mg), and **3** (t_R = 61.0 min, 4.0 mg). Finally, compounds **1** (t_R = 46.0 min, 4.5 mg), **2** (t_R = 48.5 min, 3.0 mg), and **12** (t_R = 52.0 min, 6.5 mg) were obtained from subfraction F5.2.4 (95 mg) by HPLC (0–60 min: 35% MeCN, 65 min: 100% MeCN).

1,5-dihydroxy-1-(4,5-dihydroxy-3-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,4,6-heptatrien-3-one (1): Yellow amorphous powder; UV (MeOH) λ_{max} nm (log ε) 290 (3.76), 418 (4.12); IR (KBr) ν_{max} 3416 (OH), 1665 (C=O), 1588, 1547, 1512, 1490, 1284 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 1**; HREIMS *m/z* 400.1159 $[M]^+$ (Calcd for $C_{21}H_{20}O_8$, 400.1158).

1,5-dihydroxy-1-(4,5-dihydroxy-3-methoxyphenyl)-7-phenyl-1,4,6-heptatrien-3-one (2): Yellow amorphous powder; UV (MeOH) λ_{max} nm (log ε) 295 (3.53), 398 (4.06); IR (KBr) ν_{max} 3415 (OH), 1665 (C=O), 1598, 1547, 1511, 1489, 1273 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 1**; HREIMS *m/z* 354.1105 $[M]^+$ (Calcd for $C_{20}H_{18}O_6$, 354.1103).

1-(4,5-dihydroxy-3-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione (**3**): Yellow amorphous powder; UV (MeOH) λ_{max} nm (log ε) 264 (3.74), 422 (4.27); IR (KBr) ν_{max} 3415 (OH), 2917, 1737 (C=O), 1626, 1599, 1513, 1467, 1272 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 1**; HREIMS *m/z* 384.1210 $[M]^+$ (Calcd for $C_{21}H_{20}O_7$, 384.1209).

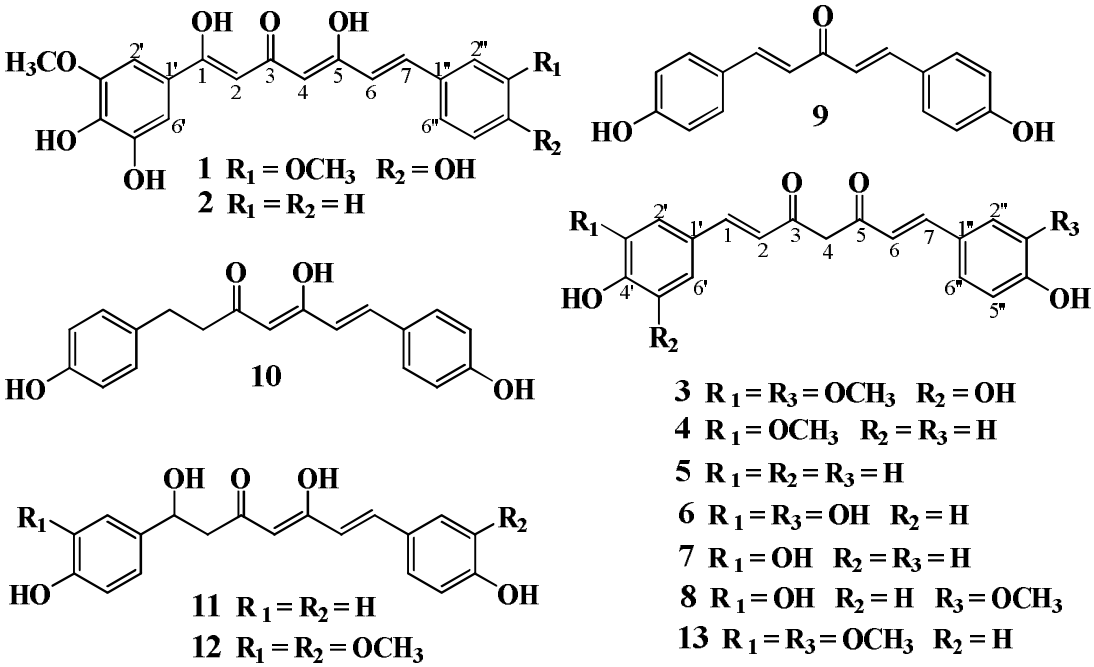

Figure 2. Chemical structures of isolated compounds

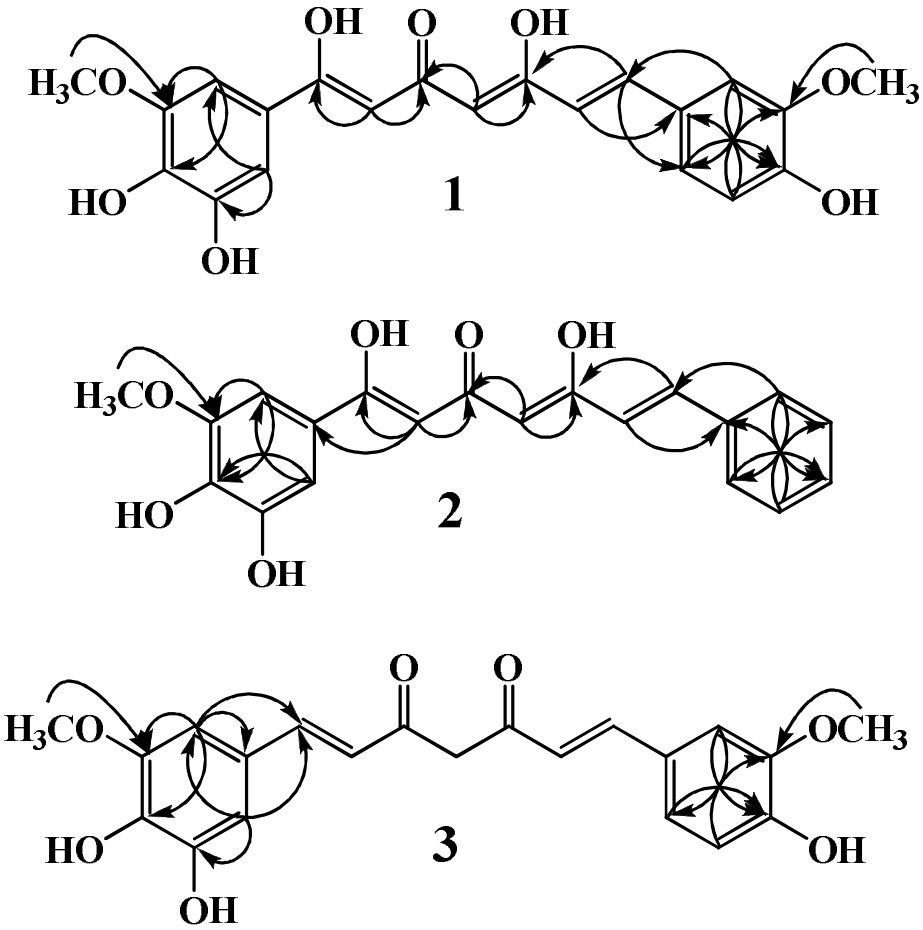

Figure 3. Key HMBC (H → C) correlations for new compound **1-3**

2.2.2 Cloning of novel H1N1 influenza NA

A full length cDNA encoding the neuraminidase of novel H1N1 influenza (A/California/08/2009(H1N1)) was constructed using a custom gene synthesis service (Nanomol, South Korea). The synthesized cDNA was subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad) for protein expression. Oseltamivir-resistant neuraminidase (H274Y mutant) was generated using a PCR-mediated site directed mutagenesis method with the forward primer GAA TGC CCC TAA TTA TTA CTA TGA GGA ATG CTC and reverse primer GAG CAT TCC TCA TAG TAA TAA TTA GGG GCA TTC, and the mutant clone was sequenced to confirm the presence of the intended mutation.

2.2.3 Viruses, cells, and expression of neuraminidase

The influenza strains A/Chicken/Korea/O1310/2001 (H9N2) and A/Sw/Kor/CAH1/04(H1N1, KCTC 11165BP) were used in this study. 293T cells (human embryonic kidney cells) and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Welgene) supplemented with 10% fetal bovine serum at 37 ^{o}C and 5% CO_2. DMEM containing 0.15 μg/ml trypsin and 5 μg/ml BSA was used as the infection medium for the MDCK cells. The 293T cells were counted and plated in 6-well plates at a density of 10^5 cells/well. After 24 hrs, the cells were transfected with plasmids containing the cDNAs using a commercial transfection kit (Welfect EX-plus, Welgene, Daegu, Korea), according to the manufacturer's instructions. Briefly, the cells were incubated in TOM along with 1.5 μg of DNA and 7.5 μg Welfect-Ex plus reagent for 6 hrs at 37 ^{o}C. After transfection, the cultures were maintained in 5% FBS-DMEM medium. At 48 hrs after post-transfection, the cells were treated with 0.02% ethylene diamine tetra acetic acid (EDTA) in PBS and harvested.

2.2.4 Influenza A (H1N1 and H9N2) neuraminidase inhibition assay

The enzyme assay was performed as previously reported with a slight modification[29]. In general, large-scale influenza virus suspension was prepared from MDCK cells infected with the influenza viruses, H1N1 and H9N2. The virus suspensions were treated with formaldehyde at a final concentration of 0.01% at 37°C for 30 min to inactivate viral infectivity. The NA activity was measured using 4-methylumbelliferyl-α-D-N-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) (Sigma, M8639) in an acetate buffer as the substrate. All compounds were dissolved in DMSO and diluted to the corresponding concentrations in MES buffer (32.5 mM 2-(N-morpholino)-ethanesulfonic acid, 4 mM $CaCl_2$, pH 6.5). The enzyme inhibitory assay was carried out in 96-well plates containing 10 μL of diluted virus supernatant (containing active influenza NA) and 10 μL isolated compound in the enzyme buffer. The mixture was incubated for 30 min at 37°C, and 30 μL 4-MU-NANA substrate per well in enzyme buffer was then added. The enzymatic reactions were carried out for 2 hrs at 37°C and then quenched by adding 150 μL of the stop solution (25% ethanol, 0.1 M glycine, pH 10.7). The fluorescence intensity of the product (4-MU) was measured using a Spectramax M2^{e} spectrofluorometer with excitation and emission wavelengths of 360 and 440 nm, respectively. The IC_{50} for reducing the NA activity was then determined, the data was analyzed using Sigmaplot 11.0 (SPCC Inc., Chicago, IL). For the enzyme kinetic study, 4-methylumbelliferone was quantified immediately without adding the stop solution.

$$\% \text{ Inhibition} = \frac{100}{1+(IC_{50}/[I])}$$

2.2.5 Novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) neuraminidase inhibition assay

The 293T cells transfected with the plasmids were harvested by a treatment with 0.02% EDTA in PBS. After washing with PBS, the cells (approximately 5×10^6 cells) were suspended in 250 μL PBS containing 3.5 mM $CaCl_2$. The suspensions were then divided into 50 μL aliquots and stored at -80°C until use. The NA inhibition assays were performed using 4-MU-NANA as the fluorescent substrate and dilutions of the samples with a NA activity equivalent to 8–10 × fluorescence units compared to the background. The tested compounds were pre-incubated with 10 μL cell suspensions in 32.5 mM MES buffer (containing 4 mM $CaCl_2$, pH 6.5) at 37°C in 30 min. After 30 min incubation, the substrate (30 μL) was added and the assays were incubated for a further 2 hrs at 37°C, and finally terminated by adding 150 μL of the stop solution (25% ethanol, 0.1 M glycin, pH 10.7). The plates were read in a Spectramax M2^e spectrofluorometer with an excitation and emission wavelength of 360 nm and 465 nm, respectively.

2.2.6 Cytopathic effect (CPE) inhibition assay

After virus was inoculated onto near confluent MDCK cell monolayers (1×10^5 cells/well) for 1 hr, the cells were replaced with DMEM containing 10 μg/mL trypsin and several compounds at different concentrations. During the cultures were incubated for 3–4 days at 37°C under 5% CO_2 atmosphere, the level of virus inhibition was determined in triplicate by adding 0.034% neutral red to each well and followed by incubation for 2 hrs at 37°C in the dark. The neutral red solution was removed and the cells were washed with PBS (pH 7.4). After adding a destaining solution (containing 1% glacial acetic acid, 49% H_2O, and 50% ethanol) to each well, the plates were incubated in the dark for 15 min at room temperature. The 50%

effective concentration (EC_{50}) was calculated by regression analysis of the absorbance at 540 nm in a microplate reader.

3. Results and Discussion

3.1 Structure determination of curcuminoids from *Curcuma longa*

A succession of chromatographic procedures (silica gel, Sephadex LH-20, RP-18, and HPLC) of the methanol extract of *C. longa* afforded thirteen compounds including three new (**1**–**3**) along with ten known (**5**–**13**) curcumin derivatives as active principles (**Figure 2**). The structure of known compounds **4**–**13** were identified to be demethoxy curcumin (**4**)[28], bisdemethoxy curcumin (**5**)[28], 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (**6**)[30], 1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (**7**)[31], 1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (**8**)[31], 1,5-bis(4-hydroxyphenyl)-1,4-pentadiene-3-one (**9**)[25], 5-hydroxy-1,7-bis(4-hydroxyphenyl)-4,6-heptadiene-3-one (**10**)[31], 1,5-dihydroxy-1,7-bis(4-hydroxyphenyl)-4,6- heptadiene-3-one (**11**) (*20*), 1,5-dihydroxy-1,7-bis(4-hydroxyl-3-methoxyphenyl)-4,6-hepta-diene-3-one (**12**)[31] and curcumin (**13**)[28] by a comparison of their physicochemical and spectroscopic data with those reported in the literature.

Compound **1** was obtained as a yellow amorphous powder with a molecular formula of $C_{21}H_{20}O_8$ from the HREIMS data ($[M]^+$, *m/z* 400.1159). The IR spectrum revealed an absorption for a hydroxy group (3416 cm^{-1}), conjugated carbonyl group (1665 cm^{-1}), and aromatic ring (1588, 1547, 1512, and 1490 cm^{-1}). The ^1H NMR spectrum (**Table 1**) showed a 1,3,4,5-tetrasubstituted benzene ring [δ_H 7.21 (1H, d, J = 2.0 Hz, H-6′), and 7.20 (1H, d, J = 2.0 Hz, H-2′)], a 1,3,4-trisubstituted benzene ring [δ_H 7.42 (1H, d, J = 2.0 Hz, H-2"), 7.22 (1H, dd, J = 8.0, 2.0 Hz, H-6"), and 6.90 (1H, d, J = 8.0 Hz, H-5")], a pair of *trans*-olefinic protons [δ_H 7.65 (1H, d, J = 16.0 Hz, H-5) and 7.12 (1H, d, J = 16.0 Hz, H-4)], two methoxy groups [δ_H 3.98 (3H, s, 3"-OCH_3) and 3.92 (3H, s, 3′-OCH_3)], and two proton on the central carbon of *β*-

diketone in their enol form δ_H 6.51 (1H, s, H-2) and 5.89 (1H, s, H-4). Consistent with the above ^{1}H NMR analysis, the ^{13}C NMR spectrum displayed signals corresponding to the methoxy groups (δ_C 56.5 and 56.4), two olefinic carbons (δ_C 139.7 and 113.6), a conjugated ketone at δ_C 186.6, two hydroxylated olefinic carbon at δ_C 186.6 and 176.1, the central carbons of *β*-diketone at δ_C 112.2 and 105.3, and twelve carbons of two aromatic rings. The protonated carbons and their bonded protons were determined unambiguously by the HSQC experiment. The HMBC correlations (**Figure 3**) from H-2 and H-4 to the carbonyl carbon at δ_C 186.6 (C-3), and H-4 to the hydroxylated olefinic carbon at δ_C 176.1 (C-5), and a olefinic carbon signal at δ_C 113.6 (C-6), from H-6 to C-5, and an aromatic carbon at δ_C 128.2 (C-1"), as well as from H-7 to the hydroxylated olefinic carbon (C-5) revealed the presence of a seven-carbon fragment (-C(OH)=CH-CO-CH=C(OH)-CH=CH-). In addition, the HMBC correlations between H-7 and two unsubstituted aromatic carbons [δ_C 111.1 (C-2") and 124.5 (C-6")] indicated that the seven-carbon fragment (-C(OH)=CH-CO-CH=C(OH)-CH=CH-) was attached directly to the 1,3,4-trisubstituted aromatic ring at C-7. Finally, the substituted pattern of two benzene rings attached at C-1 and C-7 were deduced from full assignment based on HSQC and key HMBC correlations as shown in **Figure 3**. Therefore, the structure of compound **1** was determined to be 1,5-dihydroxy-1-(4,5-dihydroxy-3-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,4,6-heptatrien-3-one.

Compound **2** was obtained as a yellow amorphous powder with a molecular formula of $C_{20}H_{18}O_6$ as deduced by HREIMS ($[M]^+$, *m/z* 354.1105). Its IR spectrum revealed the presence of a hydroxy group (3415 cm^{-1}), conjugated carbonyl group (1665 cm^{-1}) and aromatic ring (1598, 1547, 1511, and 1489 cm^{-1}). Similar to compound **1**, the ^{1}H and ^{13}C NMR spectra data (**Table 1**) showed signals for a 1,3,4,5-tetrasubstituted benzene ring, a pair of *trans*-olefinic protons, a methoxy group, and two protons on the central carbon of *β*-diketone in their enol

form. However, the second benzene ring of compound **2** is unsubstituted with the signals at δ_H 7.64 (3H, m) and 6.94 (2H, m). The HMBC correlations from H-6 to C-1" (δ_C 127.8), and from aromatic protons (H-2" and 6") to carbons [C-7 (δ_C 139.3) and C-1"] indicated this benzene ring being attached to C-7. Therefore, the structure of compound **2** was determined to be 1,5-dihydroxy-1-(4,5-dihydroxy-3-methoxyphenyl)-7-phenyl-1,4,6-heptatrien-3-one.

Compound **3** was obtained as a yellow amorphous powder. The IR spectrum showed absorption bands for hydroxy group (3415 cm^{-1}), carbonyl groups (1737, and 1626 cm^{-1}) and aromatic ring (1599, 1513, and 1467 cm^{-1}). The ^1H NMR spectrum (**Table 1**) of **3** showed signals for a 1,3,4-trisubstituted benzene ring [δ_H 7.34 (1H, s, H-2″), 7.18 (1H, d, J = 8.0 Hz, H-6″), and 6.90 (1H, d, J = 8.0 Hz, H-5″], a 1,3,4,5-quartsubstituted benzene ring [δ_H 6.88 (1H, s, H-6′), and 6.87 (1H, s, H-2′)], two pair of *trans*-olefinic protons [δ_H 7.60 (1H, d, J = 15.0 Hz, H-7), 6.72 (1H, d, J = 15.0 Hz, H-6), and 7.53 (1H, d, J = 16.0 Hz, H-1), 6.68 (1H, d, J = 15.0 Hz, H-2)], two methoxy groups [δ_H 3.92 (3H, s, OCH_3-3″) and 3.89 (3H, s, OCH_3-3′)] and the proton on the central carbon of a β-diketone δ_H 5.98 (2H, s, H-4). The ^1H and ^{13}C NMR spectroscopic data of **3** were similar to those of curcumin with an exception of an additional hydroxy group attached to aromatic ring. This was further supported by the molecular ion peak at *m/z* 384.1210 $[M]^+$ in the HREIMS which indicated the molecular formula of $C_{21}H_{20}O_7$ for **3**. The HMBC correlations between H-2″ and H-6″/C-4″; H-2′/C-1′, C-3′, and C-4′; and the methoxy protons at 3′-OCH_3/C-3′ and 3″-OCH_3/C-3″ suggested the location of substituted groups on the two aromatic rings. Finally, the position of the additional hydroxy group was inferred as C-5′ by the HMBC correlations from H-2′ and H-6′ to C-1 (δ_C 141.8), and from H-6′ to carbons of C-2′ (δ_C 103.8) and C-5′ (δ_C 149.3) (**Figure 3**). Thus, the structure of compound **3** was elucidated as 1-(4,5-dihydroxy-3-methoxyphenyl)-7-(4-hydroxy-3-

methoxyphenyl)-1,6-heptadien-3,5-dione.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for **1–3** in acetone -d_6

Position	**1**		**2**		**3**	
	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)	δ_C
1		186.6		186.2	7.53 d (15.0)	141.8
2	6.51 s	112.2	6.51 s	112.1	6.68 d (15.0)	122.5
3		186.6		186.6		184.6
4	5.89 s	105.3	5.92 s	105.4	5.98 s	101.8
5		176.1		176.1		184.6
6	7.12 d (16.0)	113.6	7.08 d (16.0)	113.4	6.72 d (15.0)	122.4
7	7.65 d (16.0)	139.7	7.64 d (16.0)	139.3	7.60 d(15.0)	141.5
1′		124.4		124.5		128.2
2′	7.20 d (2.0)	107.9	7.20 d (2.0)	107.9	6.87 s	104.8
3′		149.2		149.1		148.9
4′		146.8		146.7		146.5
5′		150.4		150.8		149.3
6′	7.21 d (2.0)	113.6	7.23 d (2.0)	113.6	6.88 s	110.3
1"		128.2		127.8		127.3
2"	7.42 d (2.0)	111.1	7.67 m	131.2	7.34 s	111.5
3"		149.0	6.94 m	116.9		149.3
4"		150.3	7.62 m	131.0		150.0
5"	6.90 d (8.0)	116.4	6.94 m	116.9	6.90 d (8.0)	116.3
6"	7.22 dd (8.0, 2.0)	124.5	7.67 m	131.2	7.18 d (8.0)	123.9
3′-OCH_3	3.92 s	56.4	3.99 s	56.5	3.89 s	56.6
3"-OCH_3	3.98 s	56.5			3.92 s	56.4

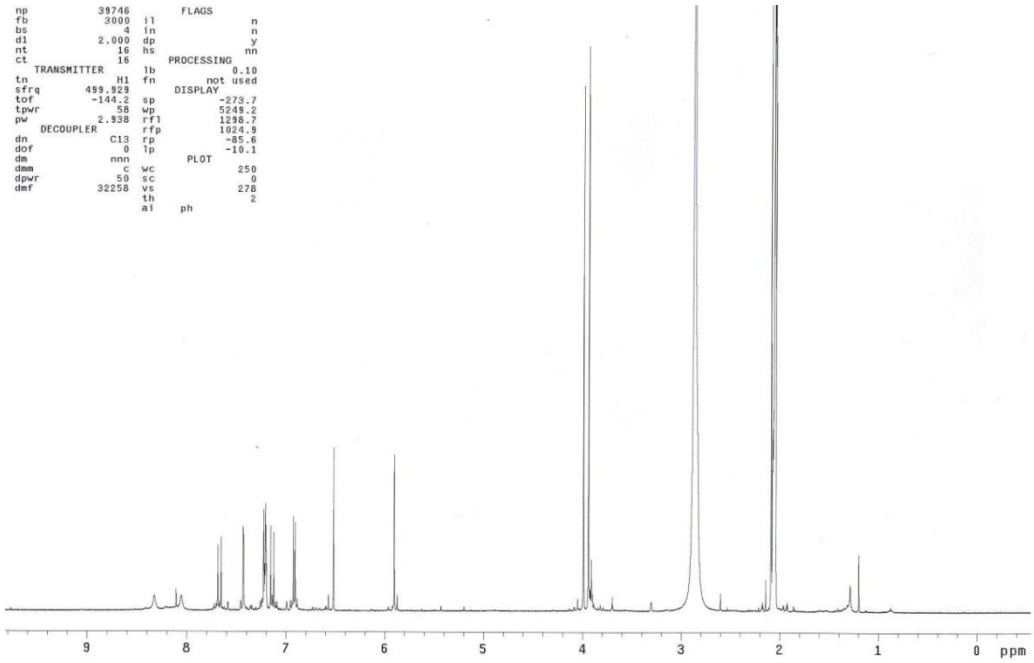

Figure 4. ^{1}H NMR (500 MHz, $CDCl_3$) of new compound **1**

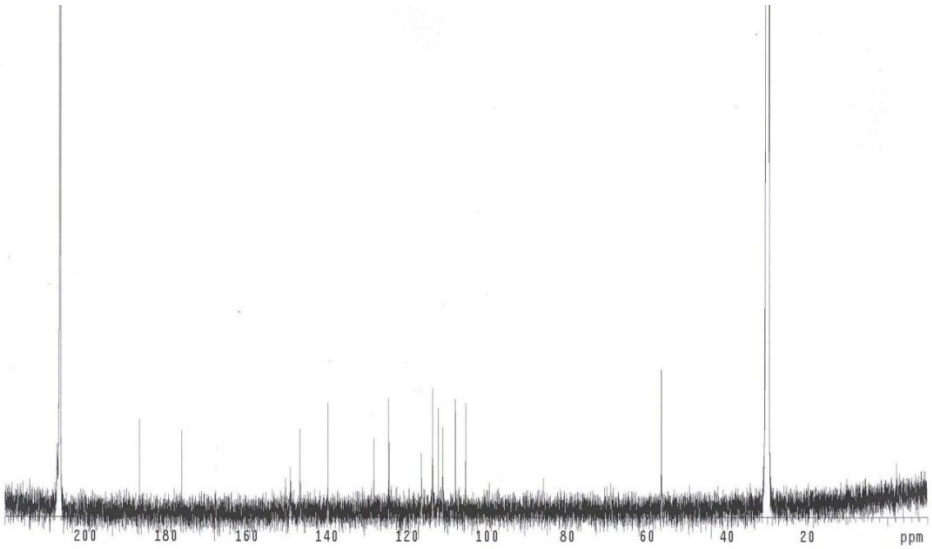

Figure 5. ^{13}C NMR (125 MHz, $CDCl_3$) of new compound **1**

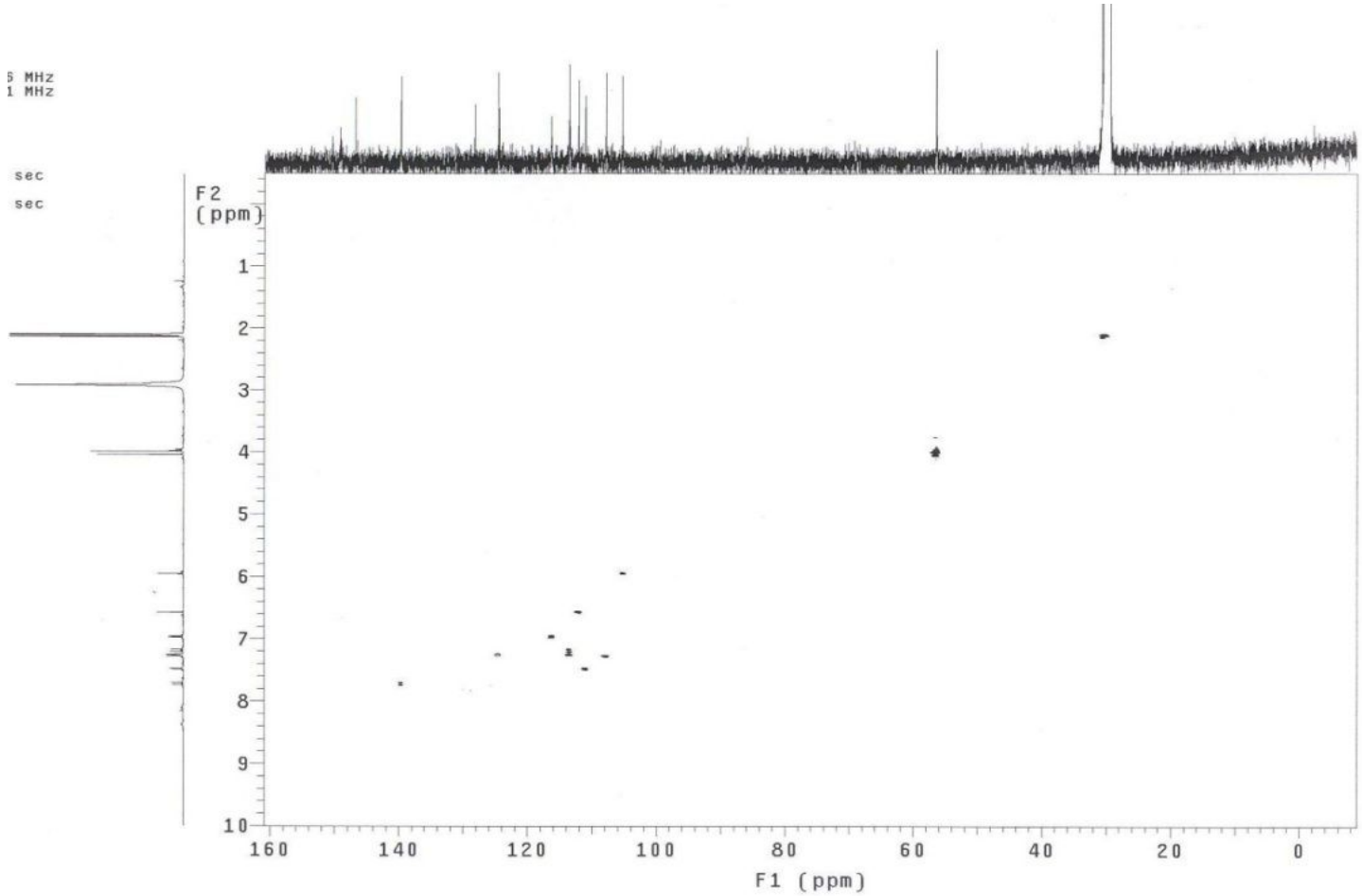

Figure 6. HSQC spectrum of new compound **1**

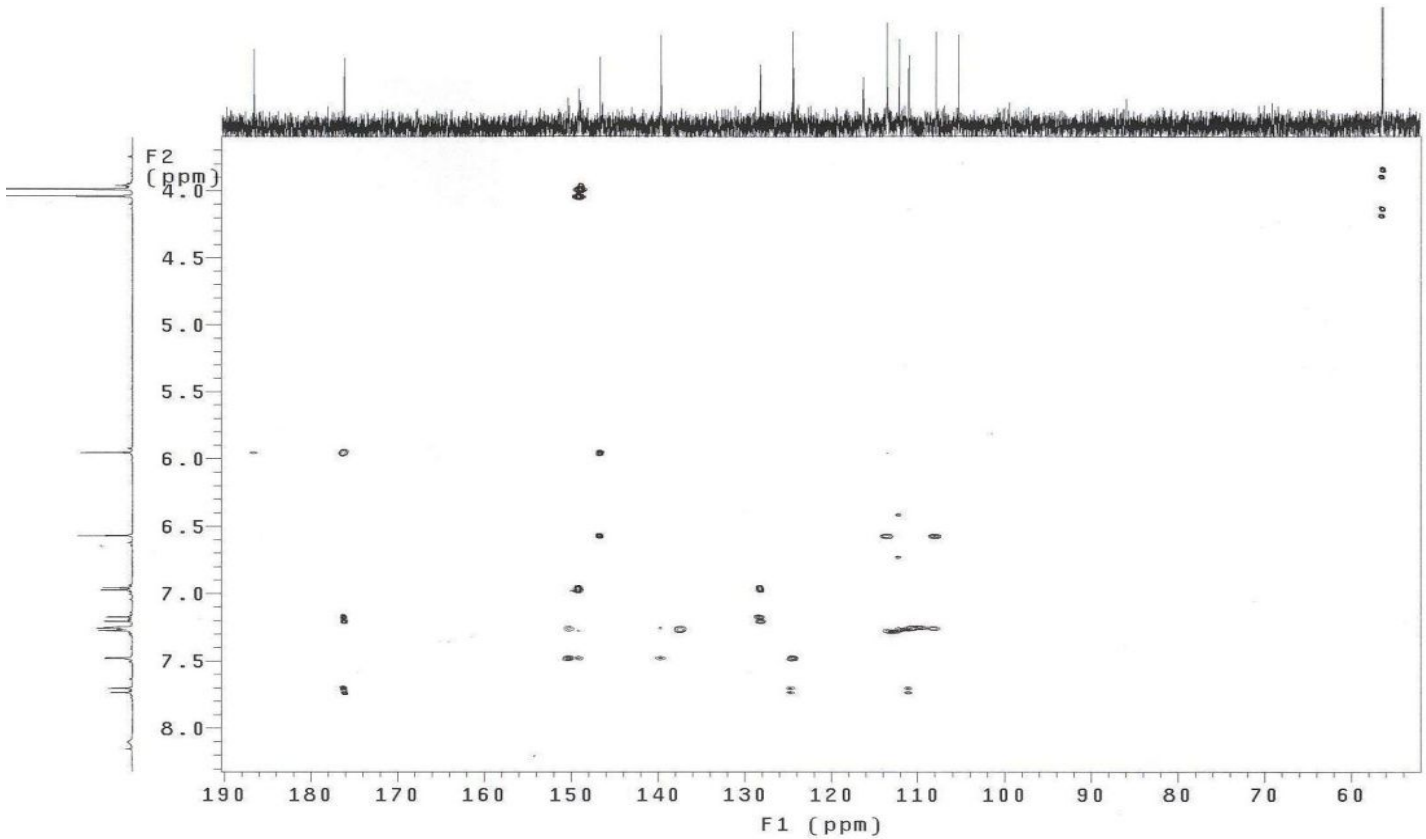

Figure 7. HMBC spectrum of new compound **1**

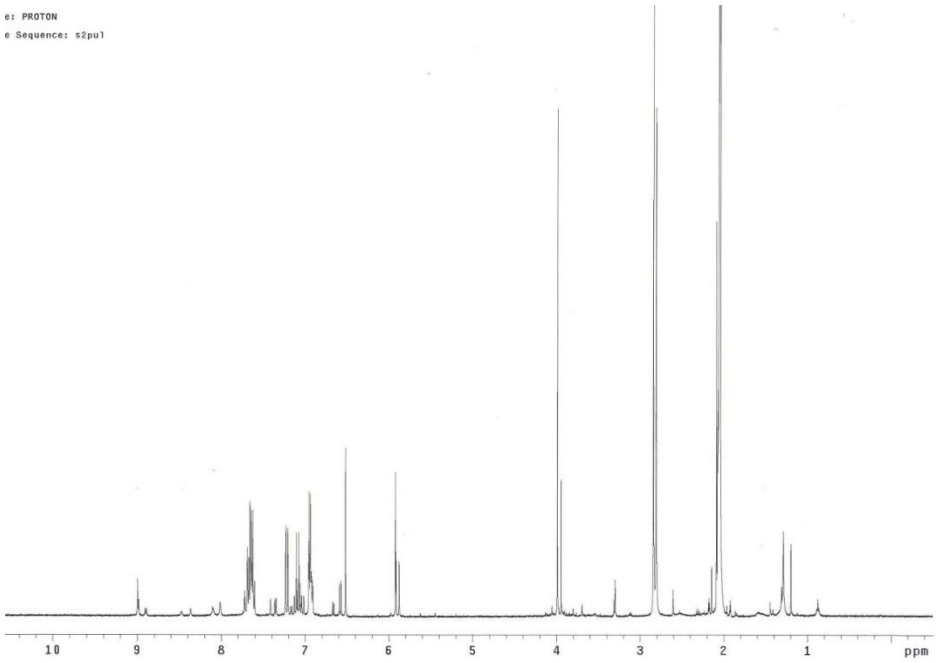

Figure 8. ^{1}H NMR (500 MHz, $CDCl_3$) of new compound **2**

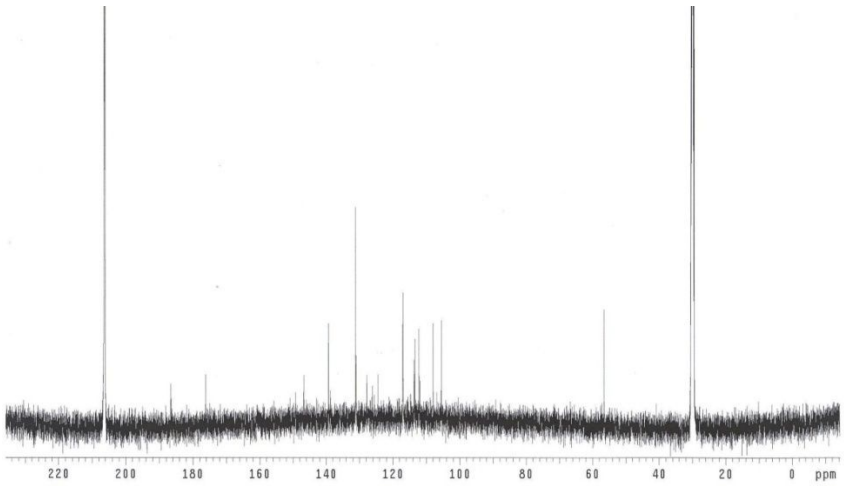

Figure 9. ^{13}C NMR (125 MHz, CD_3OD) of new compound **2**

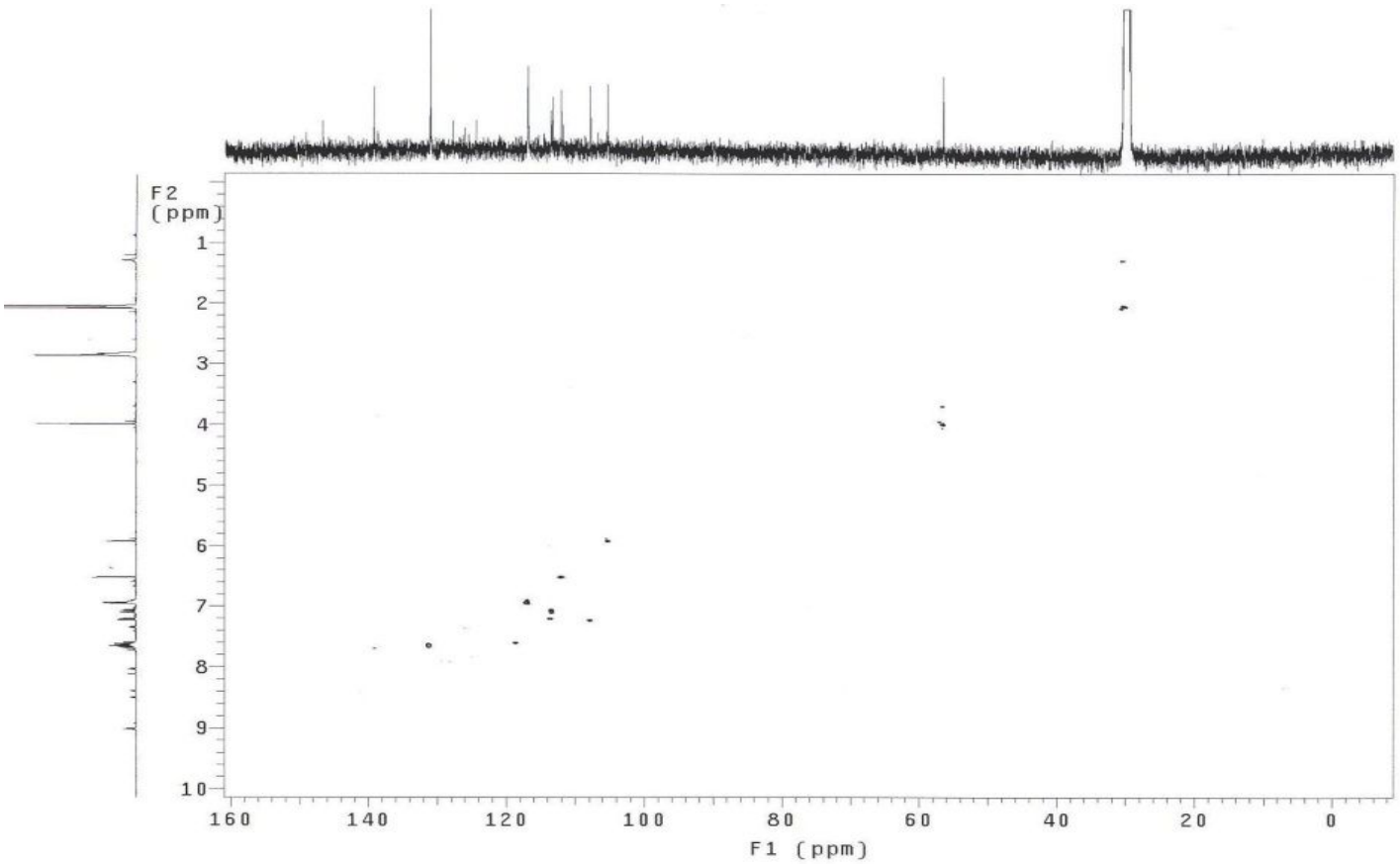

Figure 10. HSQC spectrum of new compound **2**

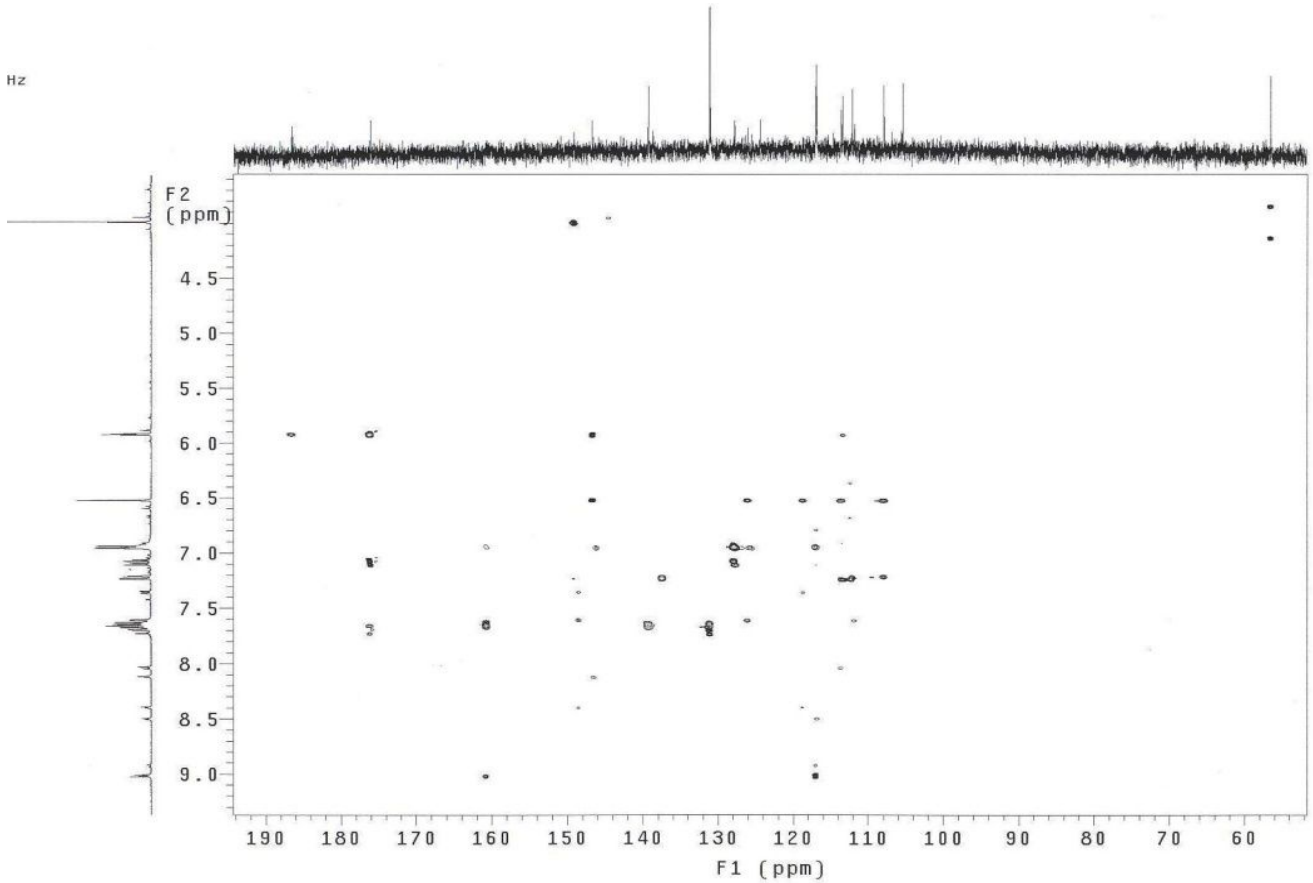

Figure 11. HMBC spectrum of new compound **2**

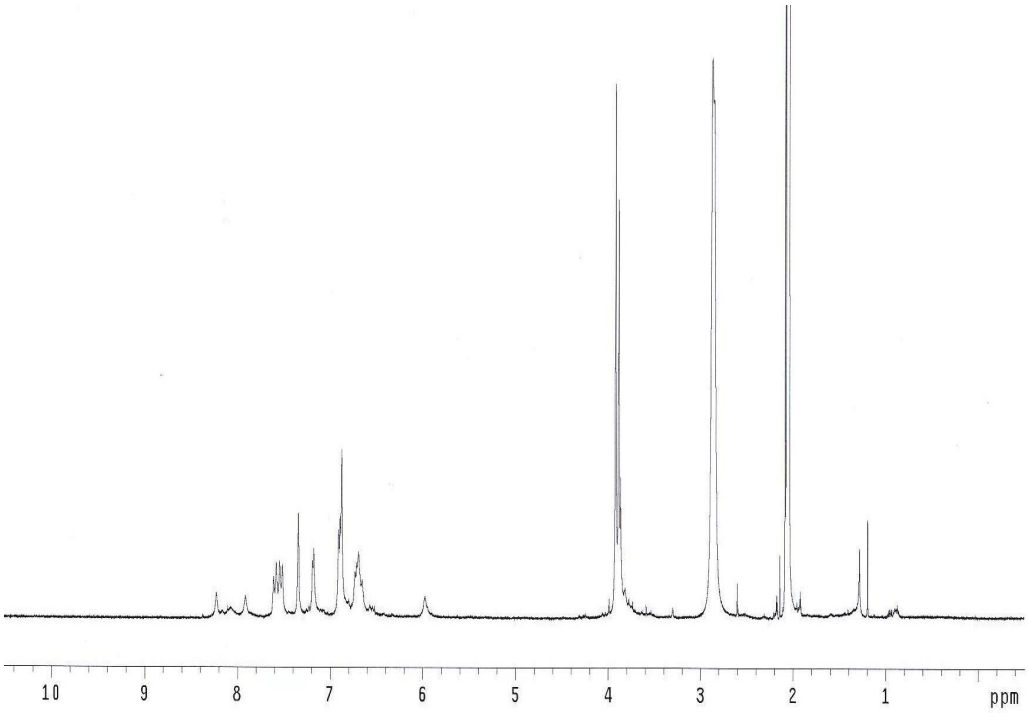

Figure 12. ^{1}H NMR (500 MHz, $CDCl_3$) of new compound **3**

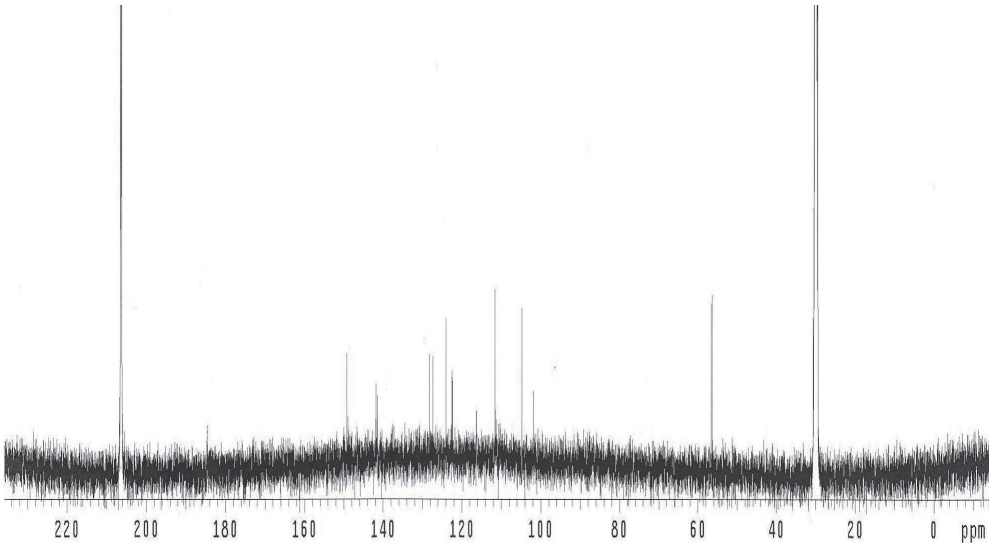

Figure 13. ^{13}C NMR (125 MHz, acetone-d_6) of new compound **3**

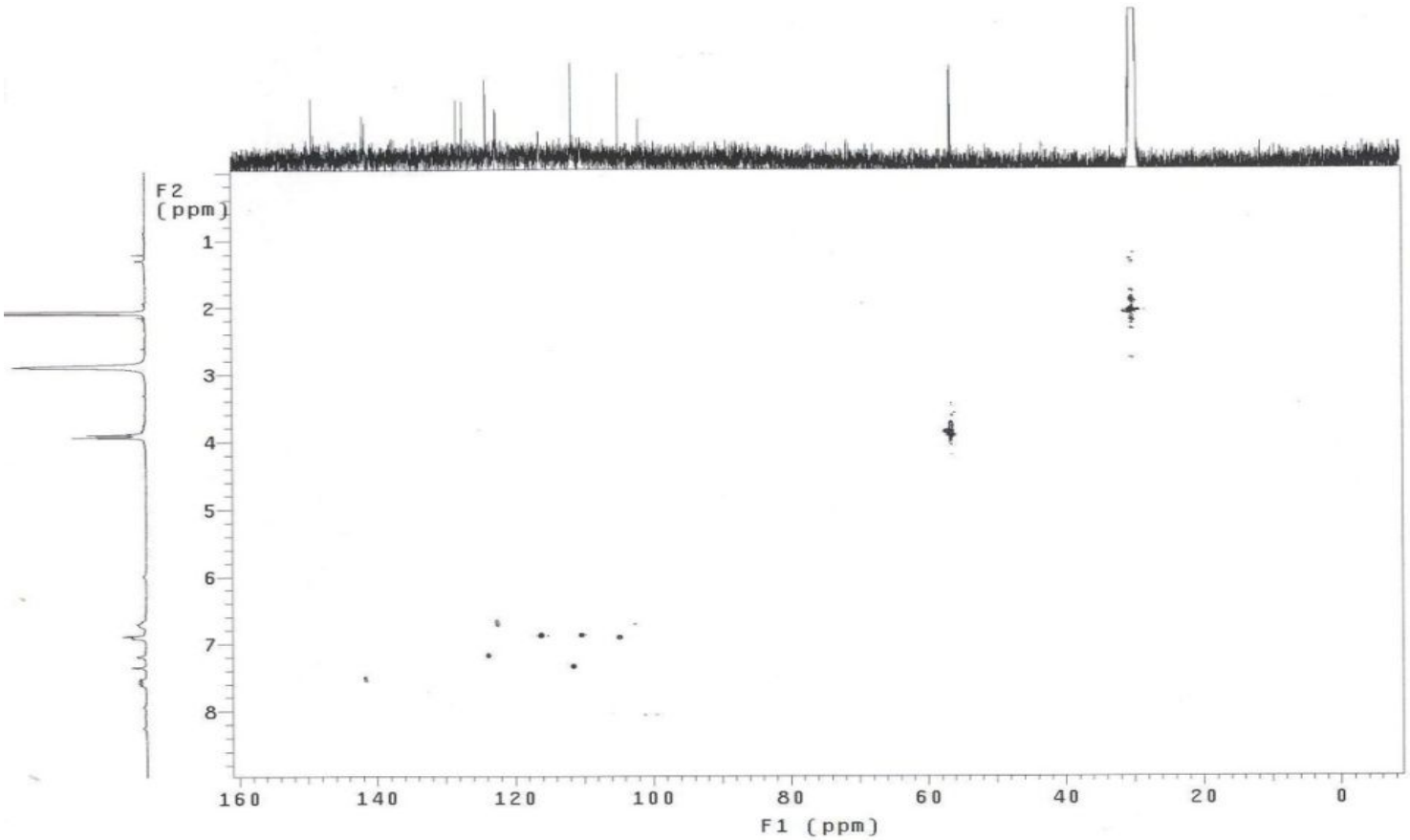

Figure 14. HSQC spectrum of new compound **3**.

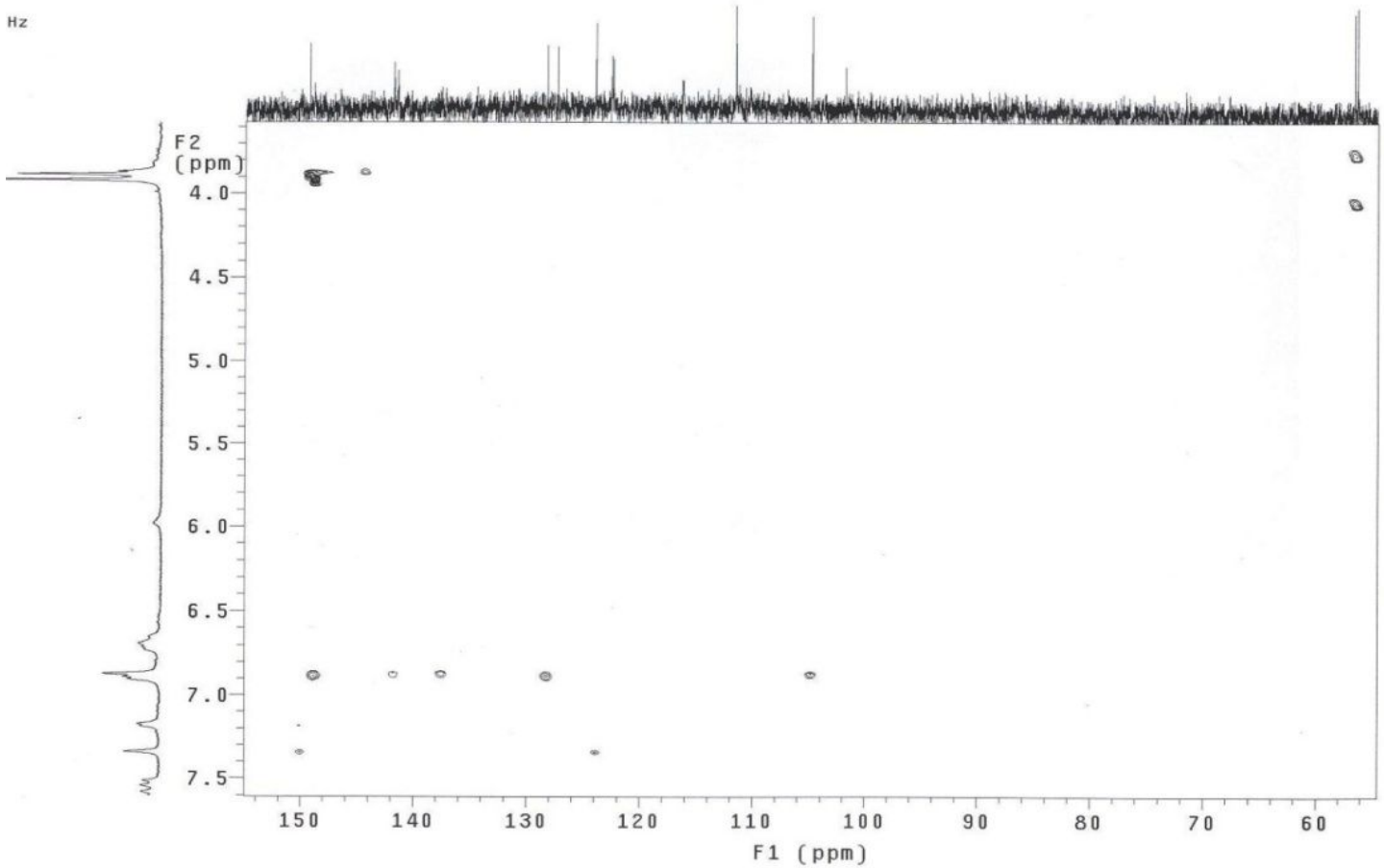

Figure 15. HMBC spectrum of new compound **3**.

3.2 Effects of the isolated compounds on NAs from two influenza viral strains, H1N1 and H9N2

The inhibitory activities of compounds **1**−**13** were assessed against NA from influenza A/PR/8/34 (H1N1) by using oseltamivir phosphate (Hoffman-La Roche Ltd, Basel, Switzerland) as a positive control. All compounds inhibited NA in a dose-dependent manner. The curcumin derivatives, **3**–**8** and **13** (IC_{50} values ranging from 8.22 ± 0.87 to 21.69 ± 1.18 μg/mL) exhibited higher activity than the other analogues (**Table 2**). The oxyaryl compounds with unsaturated -C=C-CO- unit showed a significant activity towards the NAs as in the most active compound **9** (IC_{50} 6.18 ± 0.64 μg/mL). In contrast, the presence of saturated carbons of the C_7 linker as in compounds **10**–**12** might be responsible for the decrease in activity compared to the others, e.g., **1**, **5**, and **9**. Additionally, the hydroxyl substitution of C-4 in the aryl rings might increase the NA inhibitory properties, as observed in compound **4**, **5** and **13**, compared to the structurally similar compound **6**, **7** and **8**. Interestingly, the effects on influenza virus replication of these curcuminoids also revealed IC_{50} values in the same order of magnitude against NA from another subtype H9N2 (**Table 2**).

Table 2. Inhibitory effects of compounds **1–13** on the neuraminidase activity.

Compound	H1N1	H9N2	H1N1 (WT)	H1N1 (H274Y)	
	IC_{50} (μg/mL)[a]	IC_{50} (μg/mL)[a]	IC_{50} (μg/mL)[a]	IC_{50} (μg/mL)[a]	Fold increase vs. WT
1	14.73 ± 1.42	14.26 ± 1.77	NT[c]	NT[c]	
2	15.41 ± 0.85	15.59 ± 1.38	NT[c]	NT[c]	
3	17.22 ± 1.32	18.25 ± 1.55	NT[c]	NT[c]	
4	10.25 ± 1.43	7.07 ± 0.82	4.36 ± 0.57	11.29 ± 0.55	2.59x
5	11.39 ± 0.84	9.86 ± 0.96	6.95 ± 0.92	13.74 ± 1.64	1.98x
6	18.59 ± 0.98	15.80 ± 0.92	NT[c]	NT[c]	
7	20.79 ± 1.37	25.71 ± 1.46	NT[c]	NT[c]	
8	21.69 ± 1.18	20.84 ± 1.81	NT[c]	NT[c]	
9	6.18 ± 0.64	3.77 ± 0.75	NT[c]	NT[c]	
10	20.47 ± 0.88	11.87 ± 0.94	NT[c]	NT[c]	
11	23.97 ± 1.20	18.69 ± 1.81	NT[c]	NT[c]	
12	40.17 ± 0.79	31.82 ± 1.33	NT[c]	NT[c]	
13	8.22 ± 0.87	6.17 ± 0.72	3.46 ± 0.27	6.50 ± 0.53	1.88x
Oseltamivir[b]	40.47 ± 1.29 (ng/mL)	4.29 ± 0.74 (ng/mL)	21.09 ± 1.19 (ng/mL)	5.13 ± 0.23	243.24x

[a] All compounds were examined in a set of triplicate experiments.

[b] The compound was used as the positive control.

[c] NT: not tested.

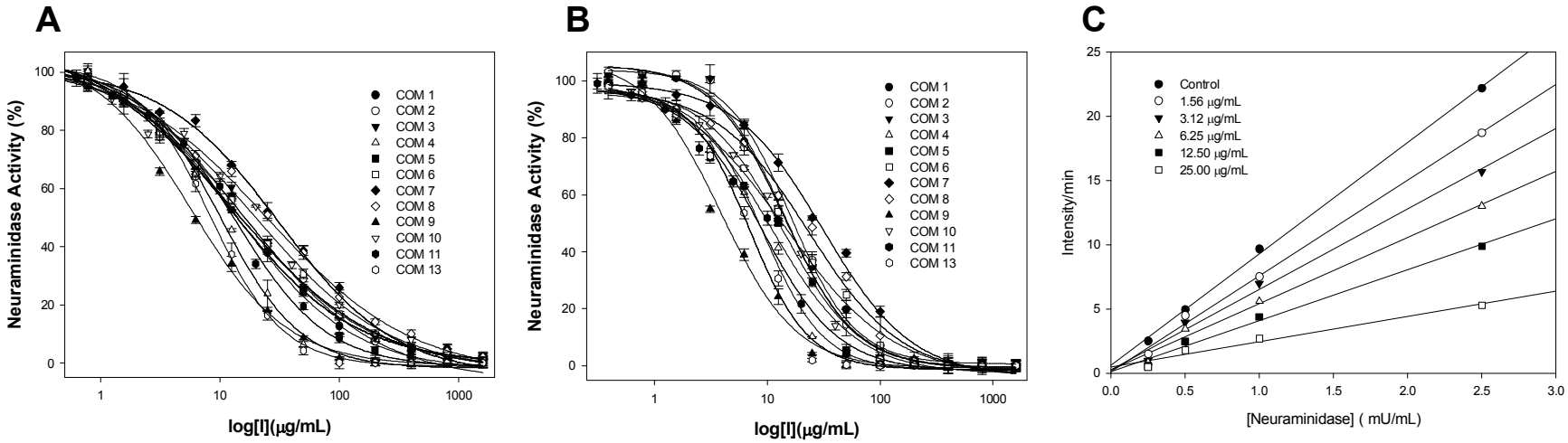

Figure 16. (A–B) Effects of compounds (**1—11** and **13**) on the activity of NA from influenza A (H1N1 and H9N2) for the hydrolysis of 4-MU-NANA at 37°C. Inhibitor concentrations are displayed on logarithmic scales. The IC_{50} is identified from the midpoint (NA activity = 50%) of the semilog plot. (C) Relationship between hydrolytic activity of NA with enzyme concentration at different concentrations of compound **9**. Concentration of compound **9** for curves from top to bottom: 1.56, 3.12, 6.25, 12.5 and 25.0 μg/mL.

3.3 Effects of the isolated compounds on NAs from novel H1N1 and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells

The major compounds **4**, **5**, and **13** were examined whether they are also effective in inhibiting NAs from the wild-type novel swine flu (WT) virus and oseltamivir-resistant virus with a H274Y mutation (22). As the results, compounds **4**, **5**, and **13** inhibited the NA derived from the wild-type with an IC_{50} of 4.36 ± 0.57, 6.95 ± 0.92, and 3.46 ± 0.27 μg/mL, respectively (**Table 2**). Interestingly, while these compounds showed inhibitory activity on recombinant NA of oseltamivir-resistant virus (slight decrease of only 1.88-2.59 fold at IC_{50}), the inhibitory activity against H274Y of oseltamivir as the positive compound was decreased notably (decrease of 243.24-fold at IC_{50} value in comparison to novel H1N1). These results suggest that although the inhibitory activities of curcuminoids on influenza NAs were weaker than those of oseltamivir, their inhibitory effects did not change in both NAs from novel H1N1 influenza and its oseltamivir-resistant (H274Y).

3.4 Inhibition pattern of the compounds on neuraminidase of H1N1

To evaluate the relative affinity of the compounds for influenza virus (H1N1) NA, we defined the inhibition mechanism of the enzyme by each compounds. As shown in **Figure 16C**, the inhibition of the tested compound was reversible because increasing the inhibitor concentration rapidly decreased enzyme activity[32]. To further study the mode of inhibition, we used both the double reciprocal Lineweaver-Burk and Dixon plots (**Figure 17**). All compounds were displayed as noncompetitive inhibitors because increasing substrate concentrations resulted in a family of lines, which intersected at a non-zero point on the *x* axis ($-K_i$) (**Figure 17A–D**). A summary of the K_i values for the tested compounds concurred with the IC_{50} values (**Table 2** and **Figure 17E–H**). Our results are consistent with a recent report indicating that

curcumin may be beneficial for the treatment of influenza virus infection by inhibiting haemagglutinin (HA) protein (*23*).

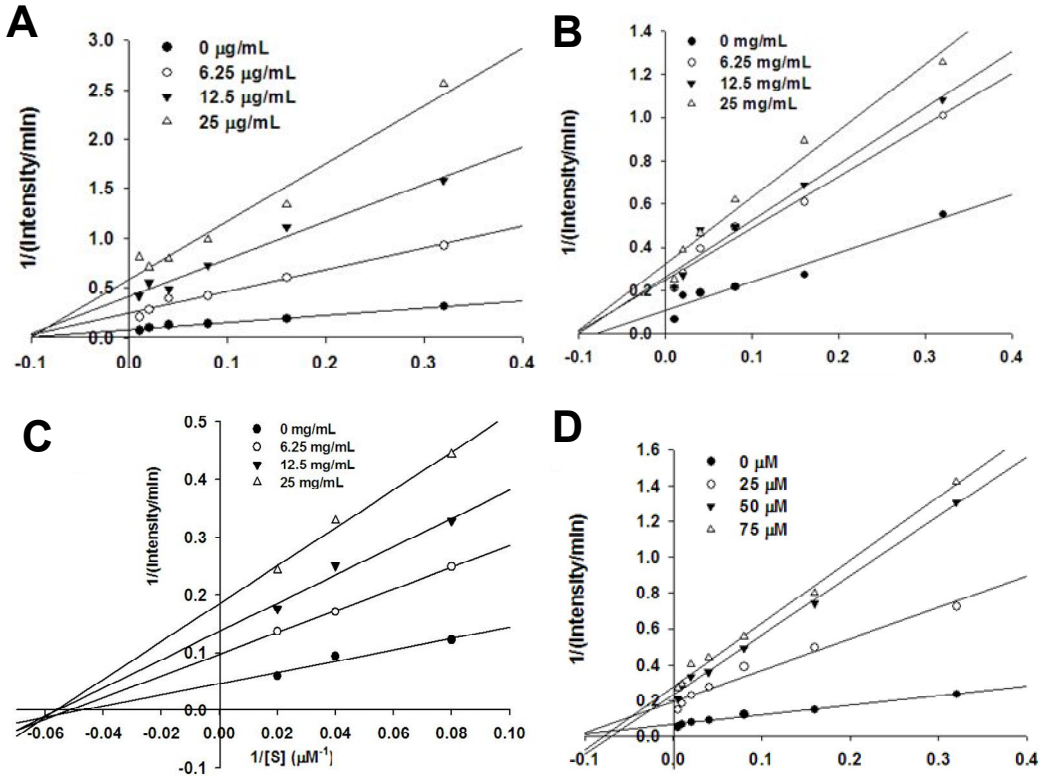

Figure 17. (A–D) Lineweaver-Burk plots for the inhibition of compounds **4**, **5**, **9** and **13** on NA from influenza A (H1N1) for the hydrolysis of substrate. Data are expressed as mean reciprocal of intensity/min for n = 3 replicates at each substrate concentration.

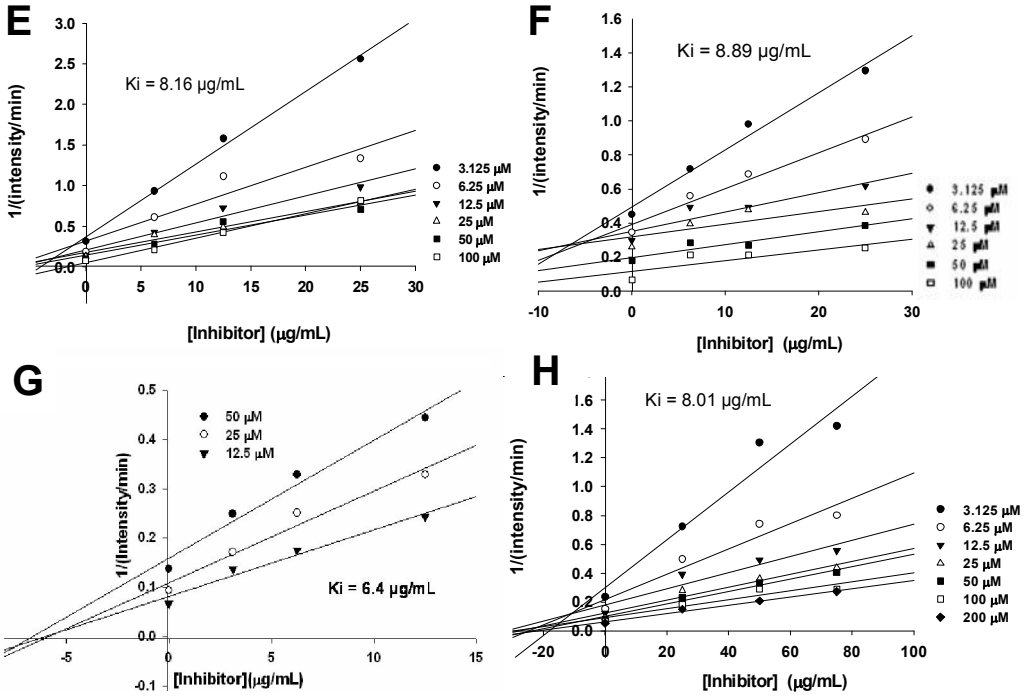

Figure 17. (E–H) Dixon plots for compounds **4**, **5**, **9** and **13** determining the inhibition constant Ki. The Ki value is determined from the negative of the x-axis value at the point of intersection of the three lines. Data are expressed as mean reciprocal of intensity/min for n = 3 replicates at each substrate concentration.

3.5 **Synergistic effect of compound 9 on NA inhibitory activity of oseltamivir**.

The noncompetitive mechanism of these compounds on NA prompted us to investigate the inhibitory effect of the combination of oseltamivir, a known competitive inhibitor, together with compound **9**. As the result, the NA inhibitory activity of oseltamivir was greatly increased, in the presence of **9** (at 1 μg/mL or 3.76 μM) (**Figure 18**). Accordingly, oseltamivir activity was increased on H9N2 and H1N1 with IC_{50} values from 6.77; 48.57 ng/mL to 1.58; 18.21 ng/mL, respectively. It is evident that oseltamivir and compound **9** act through different inhibitory mechanism, and hence maybe synergistically inhibit the NA activity by binding to different acting sites of both free enzyme and product-bound enzyme.

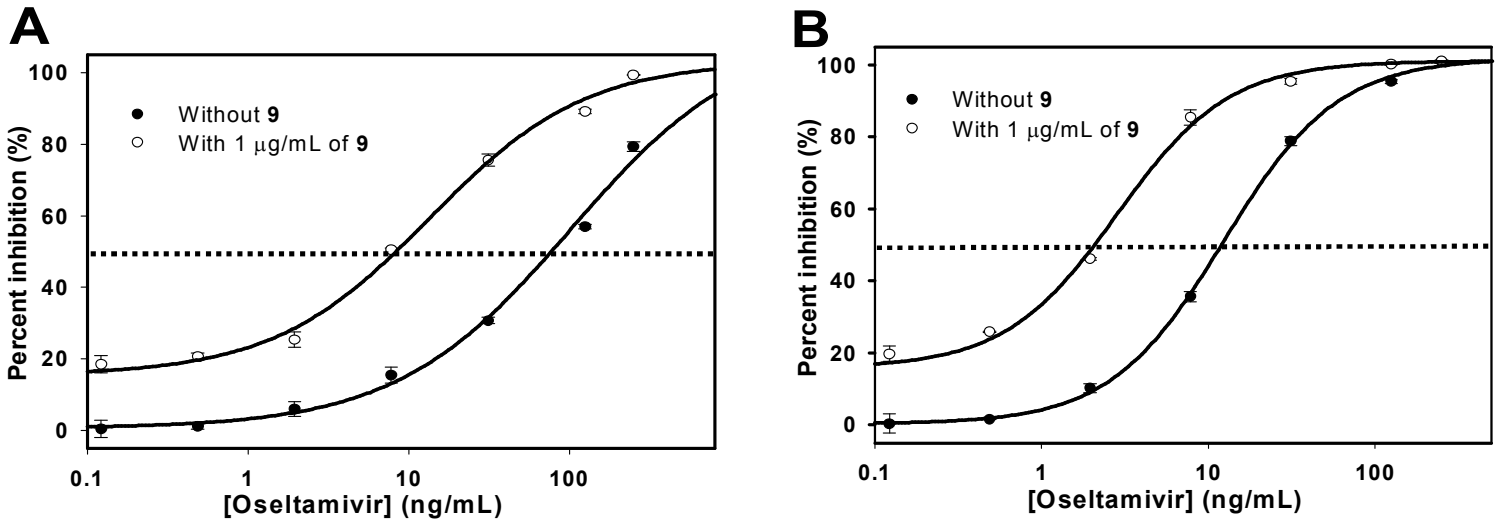

Figure 18. (A–B) Inhibition of NA from influenza A (H1N1 and H9N2) by oseltamivir in the presence or absence of compound **9**.

4. Conclusion

The emergence of drug-resistant influenza viruses and the threat of pandemics highlight the need for novel and effective antiviral agents[33]. At present, neuraminidase inhibitors are the mainstay of pharmacological strategies to fight global influenza pandemic. To date, there are two well established neuraminidase inhibitors commercially available, zanamivir and oseltamivir. The long-term of these drugs is, however, often limited by toxicity and the almost inevitable selection of drug-resistant viral mutants. Therefore, continuous research into new antiviral compounds from natural products is needed to develop new therapeutic agents in the battle against the influenza virus. This study focused on the phytochemical investigation and pharmacological evaluation of *Curcuma longa* rhizome with the target enzyme NA from two influenza viral strains (H1N1 and H9N2), novel H1N1 and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells. Three new (**1**–**3**) and ten known (**4**−**13**) curcuminoids were isolated from a methanol extract of *Curcuma longa* rhizome and shown to be effective inhibitors of neuraminidase from various H1N1 strains Although the structure-activity relationships of these compounds were not investigated thoroughly, these results suggest that curcuminoids containing oxyaryl substituent with an adjacent, unsaturated -C=C-CO- moiety, may be a new class of influenza A neuraminidase inhibitors. Furthermore, compound **9**, which has the strongest inhibitory activity on NA of novel swine influenza (H1N1), was also denoted the evidence for synergy on NA inhibition with oseltamivir. Therefore, this compound may be used as a marker component for quality control of this anti-viral botanical supplement.

Over a long period of study, curcumin has been found to exhibit various biological and pharmacological activities including anti-inflammatory, antioxidant, antimicrobial, antiviral, chemopreventive, antiangiogenic, and anticancer activities (*23*), through interactions with

various biomolecules and biochemical pathways including transcriptional factors, cell proliferation, cell survival, caspase activation, tumor suppressor, death receptor, mitochondrial, and protein kinase pathways (*12–16*, *23*, *24*). This study investigated for the first time the characteristic of curcuminoids from *C. longa* for anti-influenza viral properties. The finding may provide some important information relating the chemical structure of compounds to their activity, and also suggests that *C. longa* could be used beneficially as food supplements in the new battle of influenza A (H1N1) viruses.

5. References

1. Chand, P.; Babu, Y. S.; Bantia, S.; Chu, N.; Cole, L. B.; Kotian, P. L.; Laver, W. G.; Montgomery, J. A.; Pathak, V. P.; Petty, S. L.; Shrout, D. P.; Walsh, D. A.; Walsh, G. M., Design and synthesis of benzoic acid derivatives as influenza neuraminidase inhibitors using structure-based drug design 1. *Journal of Medicinal Chemistry* **1997,** 40, (25), 4030-4052.
2. Sakudo, A.; Baba, K.; Tsukamoto, M.; Sugimoto, A.; Okada, T.; Kobayashi, T.; Kawashita, N.; Takagi, T.; Ikuta, K., Anionic polymer, poly(methyl vinyl ether-maleic anhydride)-coated beads-based capture of human influenza A and B virus. *Bioorganic & Medicinal Chemistry* **2009,** 17, (2), 752-757.
3. Xu, X.; Zhu, X.; Dwek, R. A.; Stevens, J.; Wilson, I. A., Structural characterization of the 1918 influenza virus H1N1 neuraminidase. *J. Virol.* **2008,** 82, (21), 10493-10501.
4. Johnson, N. P.; Mueller, J., Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bull Hist Med* **2002,** 76, (1), 105-15.
5. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *New England Journal of Medicine* **2009,** 360, (25), 2605-2615.
6. Rungrotmongkol, T.; Intharathep, P.; Malaisree, M.; Nunthaboot, N.; Kaiyawet, N.; Sompornpisut, P.; Payungporn, S.; Poovorawan, Y.; Hannongbua, S., Susceptibility of antiviral drugs against 2009 influenza A (H1N1) virus. *Biochemical and Biophysical Research Communications* **2009,** 385, (3), 390-394.
7. Neuraminidase inhibitors as antiviral agents. *Current Drug Targets - Infectious Disorders* **2005,** 5, 401-409.
8. Air, G. M.; Ghate, A. A.; Stray, S. J., Influenza neuraminidase as target for antivirals. in

Advances in Virus Research, Karl Maramorosch, F. A. M.; Aaron, J. S., Eds. Academic Press: 1999; Vol. Volume 54, pp 375-402.

9. Smith, P. W.; Sollis, S. L.; Howes, P. D.; Cherry, P. C.; Starkey, I. D.; Cobley, K. N.; Weston, H.; Scicinski, J.; Merritt, A.; Whittington, A.; Wyatt, P.; Taylor, N.; Green, D.; Bethell, R.; Madar, S.; Fenton, R. J.; Morley, P. J.; Pateman, T.; Beresford, A., Dihydropyrancarboxamides related to zanamivir: a new series of inhibitors of influenza virus Sialidases. 1. Discovery, Synthesis, Biological Activity, and structure–activity relationships of 4-guanidino- and 4-amino-4H-pyran-6-carboxamides. *Journal of Medicinal Chemistry* **1998,** 41, (6), 787-797.
10. Gubareva, L. V.; Kaiser, L.; Hayden, F. G., Influenza virus neuraminidase inhibitors. *The Lancet* **2000,** 355, (9206), 827-835.
11. von Itzstein, M.; Wu, W. Y.; Kok, G. B.; Pegg, M. S.; Dyason, J. C.; Jin, B.; Van Phan, T.; Smythe, M. L.; White, H. F.; Oliver, S. W.; et al., Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **1993,** 363, (6428), 418-23.
12. Hurt, A. C.; Holien, J. K.; Parker, M. W.; Barr, I. G., Oseltamivir resistance and the H274Y neuraminidase mutation in seasonal, pandemic and highly pathogenic influenza viruses. *Drugs* **2009,** 69, (18), 2523-2531 10.2165/11531450-000000000-00000.
13. Bucher, D., and P. Palese. , The biologically active proteins of influenza virus: neuraminidase,. *In E. D. Kilbourne (ed.)* **1975.**, p. 83-123.
14. Monto, A. S.; McKimm-Breschkin, J. L.; Macken, C.; Hampson, A. W.; Hay, A.; Klimov, A.; Tashiro, M.; Webster, R. G.; Aymard, M.; Hayden, F. G.; Zambon, M., Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. *Antimicrob. Agents Chemother.* **2006,** 50, (7), 2395-2402.
15. Ammon, H. P.; Wahl, M. A., Pharmacology of *curcuma longa. Planta Med* **1991,** 57, (1),

1-7.

16. Gopalan, B.; Goto, M.; Kodama, A.; Hirose, T., Supercritical carbon dioxide extraction of turmeric (*Curcuma longa*). *Journal of Agricultural and Food Chemistry* **2000,** 48, (6), 2189-2192.

17. He, X.-G.; Lin, L.-Z.; Lian, L.-Z.; Lindenmaier, M., Liquid chromatography-electrospray mass spectrometric analysis of curcuminoids and sesquiterpenoids in turmeric (*Curcuma longa*). *Journal of Chromatography A* **1998,** 818, (1), 127-132.

18. Tomoda, M.; Gonda, R.; Shimizu, N.; Kanari, M.; Kimura, M., A reticuloendothelial system activating glycan from the rhizomes of *curcuma longa*. *Phytochemistry* **1990,** 29, (4), 1083-1086.

19. Leonel, M.; Sarmento, S. B. S.; Cereda, M. P., New starches for the food industry: *Curcuma longa* and *Curcuma zedoaria*. *Carbohydrate Polymers* **2003,** 54, (3), 385-388.

20. Simon, A.; Allais, D. P.; Duroux, J. L.; Basly, J. P.; Durand-Fontanier, S.; Delage, C., Inhibitory effect of curcuminoids on MCF-7 cell proliferation and structure-activity relationships. *Cancer Lett* **1998,** 129, (1), 111-6.

21. Park, S. Y.; Kim, D. S., Discovery of natural products from *Curcuma longa* that protect cells from beta-amyloid insult: a drug discovery effort against alzheimer's disease. *J Nat Prod* **2002,** 65, (9), 1227-31.

22. Reddy, A. C.; Lokesh, B. R., Effect of curcumin and eugenol on iron-induced hepatic toxicity in rats. *Toxicology* **1996,** 107, (1), 39-45.

23. Miriyala, S.; Panchatcharam, M.; Rengarajulu, P., Cardioprotective effects of curcumin. *Adv Exp Med Biol* **2007,** 595, 359-77.

24. Portes, E.; Gardrat, C.; Castellan, A., A comparative study on the antioxidant properties of tetrahydrocurcuminoids and curcuminoids. *ChemInform* **2007,** 38, (50).

25. Masuda, T.; Jitoe, A.; Isobe, J.; Nakatani, N.; Yonemori, S., Anti-oxidative and anti-inflammatory curcumin-related phenolics from rhizomes of curcuma domestica. *Phytochemistry* **1993,** 32, (6), 1557-1560.

26. Du, Z.-Y.; Bao, Y.-D.; Liu, Z.; Qiao, W.; Ma, L.; Huang, Z.-S.; Gu, L.-Q.; Chan, A. S. C., Curcumin analogs as potent aldose reductase inhibitors. *Archiv der Pharmazie* **2006,** 339, (3), 123-128.

27. De Clercq, E., Antiviral agents active against influenza A viruses. *Nat Rev Drug Discov* **2006,** 5, (12), 1015-1025.

28. Jayaprakasha, G. K.; Jagan Mohan Rao, L.; Sakariah, K. K., Improved HPLC method for the determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin. *Journal of Agricultural and Food Chemistry* **2002,** 50, (13), 3668-3672.

29. Hung, H.-C.; Tseng, C.-P.; Yang, J.-M.; Ju, Y.-W.; Tseng, S.-N.; Chen, Y.-F.; Chao, Y.-S.; Hsieh, H.-P.; Shih, S.-R.; Hsu, J. T. A., Aurintricarboxylic acid inhibits influenza virus neuraminidase. *Antiviral Research* **2009,** 81, (2), 123-131.

30. Venkateswarlu, S.; Ramachandra, M. S.; Subbaraju, G. V., Synthesis and biological evaluation of polyhydroxycurcuminoids. *Bioorganic & Medicinal Chemistry* **2005,** 13, (23), 6374-6380.

31. Li, W.; Wang, S.; Feng, J.; Xiao, Y.; Xue, X.; Zhang, H.; Wang, Y.; Liang, X., Structure elucidation and NMR assignments for curcuminoids from the rhizomes of *curcuma longa. Magnetic Resonance in Chemistry* **2009,** 47, (10), 902-908.

32. Dao, T.-T.; Tung, B.-T.; Nguyen, P.-H.; Thuong, P.-T.; Yoo, S.-S.; Kim, E.-H.; Kim, S.-K.; Oh, W.-K., C-Methylated flavonoids from *cleistocalyx operculatus* and their inhibitory effects on novel influenza A (H1N1) neuraminidase. *Journal of Natural Products* **2010**, 73, 1636–1642.

33. Zaraket, H.; Saito, R.; Sato, I.; Suzuki, Y.; Li, D.; Dapat, C.; Caperig-Dapat, I.; Oguma, T.; Sasaki, A.; Suzuki, H., Molecular evolution of human influenza A viruses in a local area during eight influenza epidemics from 2000 to 2007. *Archives of Virology* **2009,** 154, (2), 285-295.

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논문제목	한글: 울금 (*Curcuma longa* L.)로부터 신종 인플루엔자 A (H1N1) 뉴라미니다제 저해제인 커큐미노이드 영문: New Influenza A (H1N1) Neuraminidase Inhibitory Curcuminoids from *Curcuma longa* L.				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함. (다만, 저작물의 내용 변경은 금지함.)
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의 여부: 동의 (0) 반대 ( )

2011 년 2월 22일

저작자: 부이탄텅 (인)

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