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Thesis for Master Degree

C-methylated Flavonoids from *Cleistocalyx operculatus* and Their Inhibitory Effects on Novel Influenza A (H_1N_1) Neuraminidase

Chosun University Graduate School

Department of Pharmacy

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조선대학교 대학원

약학과

응구엔띠녹안

This thesis is examined and approved for Nguyen Thi Ngoc Anh's master degree

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2010년 08월

Chosun University Graduate School

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List of Abbreviations

[α]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)

베트남 식물 *Cleistocalyx operculatus* 로부터 분리한 메틸 연결된 플라보노이드 및 그들의 신종플루 뉴라미니데이즈에 대한 저해활성

응구엔띠녹안
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조류 및 돼지 인플루엔자와 신종플루의 방어대책에 시급성을 감안하여 효과가 확인되면 바로 적응이 가능한 식품 및 약용식물을 대상으로 하여 조류 및 돼지 인플루엔자와 신종플루에 대한 뉴라미니데이즈 저해물질을 탐색하고자 하였다. 각 종 자생식물 및 한약재를 채집하여 조류 및 돼지 인플루엔자 바이러스의 배양액을 사용하거나 신종플루 및 타미플루 내성 신종플루 바이러스의 뉴라미니데이즈를 클로닝한 후 이들을 세포내로 도입하여 뉴라미니데이즈 효소에 대한 저해 작용을 조사하던 중 베트남 식물인 *Cleistocalyx operculatus* 의 메탄올 추출물에서 분리한 네개의 신 물질과 열개의 알려진 메틸 연결된 플라보노이드를 분리 하여 인플루엔자 H_1N_1 뉴라미니데이즈 저해물질 실험을 하였다. 물질의 구조 분석은 각종 스펙트럼 데이터로 확인 하였다. 흥미롭게도 화합물 **4**, **5**, **8**, **14** 물질은 찰콘계 골격 구조로 바이러스 뉴라미니다제인 두가지 인플루엔자 바이러스 종인 H_1N_1, H_9N_2 에 효과가 좋은 걸로 나타났다. 더욱이 신규화합물인 화합물 **4**는 조류 및 돼지 인플루엔자에 IC_{50} 값 각각 2.56과

1.04 ug/ml 로서 가장 효과가 좋은 걸로 판명되었다. 이러한 결론으로 베트남 식물인 *C. operculatus* 에서 분리한 메틸 연결된 플라보노이드는 인플루엔자 H_1N_1, 뉴라미니다제 억제물로 효과가 있다는 것을 확인 할 수 있었다.

ABSTRACT

C-methylated Flavonoids from *Cleistocalyx operculatus* and Their Inhibitory effects on Novel Influenza A (H_1N_1) Neuraminidase

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As part of an ongoing study focused on the discovery of anti-influenza agents from plants, four new (**1**–**4**) and ten known (**5**−**14**) C-methylated flavonoids were isolated from a methanol extract of *Cleistocalyx operculatus* buds (nu voi) using an influenza H_1N_1 neuraminidase inhibition assay. The structures of the new compounds were identified by spectroscopic data analyses. Interestingly, compounds **4**, **5**, **8**, and **14** with a chalcone skeleton exhibited significant inhibitory effect on the viral neuraminidases from two influenza viral strains, H_1N_1 and H_9N_2. Furthermore, the new compound **4** showed the strongest inhibitory activity against the neuraminidases from novel influenza H_1N_1 (WT) and oseltamivir-resistant novel H_1N_1 (H274Y mutant) expressed in 293T cells with an IC_{50} of 2.56 and 1.04 μg/mL, respectively. In addition, these compounds behaved as noncompetitive inhibitors in kinetic studies. These results suggest that C-methylated flavonoids from *C. operculatus* may be used as neuraminidase inhibitors for influenza H_1N_1.

1. Introduction

1.1 Influenza A (H_1N_1) virus

Influenza viruses were responsible for major devastating worldwide epidemics in the human population, causing enormous economic, human, and healthcare costs. 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 (*1*). Type A viruses account for all of the human pandemics of the last century: the 1918 H_1N_1 "Spanish," the 1957 H_2N_2 "Asian," and the 1968 H_3N_2 "Hong Kong" influenza viruses (*2*). 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 (*3*). Since April 2009, a novel swine-origin influenza A (H_1N_1) 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 (H_1N_1) virus (*4*, *5*). 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 (*6*).

1.2 Neuraminidase inhibitors

One of the key surface proteins of the influenza virus which are important for viral replication and spread of influenza virus is neuraminidase (NA) also known as sialidase. This tetrameric protein is a glycohydrolase that catalyzes the cleavage of terminal-ketosidically linked sialic acids from a large variety of glycoproteins, glycolipids, and oligosaccharides (*7*, *8*). NA is an attractive target for antiviral strategy because of its essential role in the pathogenicity of many respiratory viruses. It removes sialic acid from the surface of infected cells and virus particles, thereby preventing viral self-aggregation and promoting efficient viral spread; NA also plays a role in the initial penetration of the mucosal lining of the respiratory tract (*6*). Until now, several potent and specific inhibitors of this enzyme have been developed, and two (zanamivir and oseltamivir) have been approved for human use. 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. 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 (*9*).

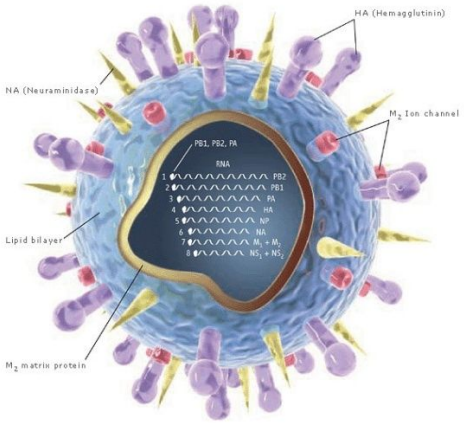


Figure 1. Structural diagram of the Influenza virus

Oseltamivir in particular, are the drugs of choice against seasonal influenza, and are stockpiled as the primary mitigating strategy for pandemic influenza containment and control. Recently, however, significant levels of oseltamivir-resistant influenza A (H_1) seasonal influenza viruses have also been encountered, which has been associated with a single amino acid change in the viral neuraminidase (H274Y) (*10*). Due to the high prevalence of H_1N_1 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 (*11*). Therefore, additional antiviral approaches are urgently needed and it is desirable to develop the novel antiviral medicines which overcome the drug resistance.

1.3 Cleistocalyx operculatus (Roxb.) Merr and Perry (Myrtaceae)

C. operculatus is a medium sized tree (6–12 meters in height) that is distributed widely in tropical Asia. Its leaves have been used in traditional medicine for the treatment of gastric ailments, while the buds, commonly called “nu voi”, have been used since ancient times to

produce various beverages, as an antiseptic agent and a tonic drink for fever patients in Vietnam and southern China (*12*). *C. operculatus* extract exhibits strong protective effects on the lipid peroxidation in rat liver microsomes (*13*). It is also considered to be a promising material for preventing and treating diabetes (*14*). Previous phytochemical attention has led to the characterization of some triterpenoids, flavonoids, and essential oils from the buds, which possess antioxidant and anti-inflammatory activity (*15–18*). Recently, the ethanol extract of the buds of this plant exhibited inhibitory activity against a wide range of bacterial strains (*19*). However, there are no reports of *C. operculatus* exhibiting influenza neuraminidase inhibitory activity.

During the course of an anti-influenza screening program on natural products, the methanol extract of *C. opercurlatus* was found to exhibit potential NA inhibitory properties. This prompted us to phytochemically examine the molecular constituents responsible for the NA inhibitory activity using an *in vitro* assay as bioactivity-guided fractionation. This paper describes the isolation, structural elucidation, and antiviral activity of these compounds on NAs

from two influenza viral strains, H_1N_1 and H_9N_2, as well as from both novel H_1N_1 (WT) and oseltamivir-resistant novel H_1N_1 (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 buds of *Cleistocalyx operculatus* (Roxb.) Merr. and Perry were purchased at the Dong Xuan herbal market, Hanoi, Vietnam, in February 2009 and were identified botanically by Dr. Nguyen Bich Thu, National Institute of Medicinal Materials, Hanoi, Vietnam. A voucher specimen (NIMM2009–05) was deposited at the herbarium of the National Institute of Medicinal Materials, Hanoi, Vietnam.

2.2 Methods

2.2.1 Extraction and Isolation

The dried buds of *C. operculatus* (1.5 kg) were extracted with methanol (4 L × 2 times) at room temperature for one week. The combined methanol extract was then concentrated to yield a dry residue (170 g). This crude extract was suspended in water (2 L) and partitioned successively with n-hexane (3 × 1.5 L), ethyl acetate (3 × 1.5 L), and n-butanol (3 × 1.5 L). The ethyl acetate fraction (75 g), which exhibited strong influenza NA inhibitory activity, was chromatographed over a silica gel column (10 × 30 cm; 63–200 μm particle size) eluting with gradient solvent n-hexane/EtOAc (19:1, 18:2…1:19, each 2.5 L) to yield nine fractions (F1: 10600 mg; F2: 3500 mg; F3: 2500 mg; F4: 4200 mg; F5: 5570 mg; F6: 3600 mg; F7: 2450 mg; F8: 5550 mg; F9: 10600 mg) based on the TLC profile. Fraction F3 was chromatographed over a Sephadex LH-20 column (7 × 40 cm) using MeOH as the eluting solvent to yield compound 5 (750 mg). Fraction F4 was applied to a RP-18 column (7 × 30 cm; 40–63 μm particle size) eluting with a stepwise gradient of MeOH/H_2O (2:1 to 10:1) to afford five subfractions (F4.1–F4.5). F4.2 (160 mg) was separated by HPLC [Optima Pak C18 column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeOH in H_2O containing 0.1% formic acid (0–65 min: 63% MeOH, 65–70 min: 100% MeOH, 70–80 min: 100% MeOH); flow rate 2 mL/min; UV detection at 205 and 254 nm] to give new compound **1** (tR = 45.0 min, 3.5 mg), 5-hydroxy-7-methoxy-6,8-dimethylflavanone (**6**) (t_R = 49.0 min, 13.5 mg) and 7-hydroxy-5-methoxy-6,8-dimethylflavone (**7**) (t_R = 63.0 min, 4.5 mg). From subfraction F4.3 (220 mg), 2′,4′-dihydroxy-3′-methyl-6′-methoxychalcone (**8**) (t_R = 31.0 min, 19.5 mg), 6-formyl-8-methyl-7-O-methylpinocembrin (**9**) (t_R = 45.0 min, 9.5 mg) and (2S)-8-formyl-5-hydroxy-7-methoxy-6-methylflavanone (**10**) (t_R = 48.0 min, 14.0 mg) were separated by HPLC (0–55 min: 72% MeOH, 60 min: 100% MeOH). Fraction F5 was chromatographed over a Sephadex LH-20

column (7 × 30 cm) using MeOH as the eluting solvent to give four subfractions (F5.1–F5.4). F5.3 (2600 mg) was fractionated continuously into five subfractions (F5.3.1–F5.3.5) using a RP-18 column (5 × 30 cm; 40–63 μm particle size) with a stepwise gradient of MeOH/H_2O (1:1 to 10:1). Subfraction F5.3.2 (150 mg) was further separated by HPLC (0–45 min: 58% MeOH, 50 min: 100% MeOH), which led to the isolation of new compound **2** (t_R = 28.0 min, 4.5 mg) and 7-hydroxy-5-methoxy-8-methylflavanone (**11**) (t_R = 40.0 min, 14 mg). Using HPLC (0–65 min: 62% MeOH, 70 min: 100% MeOH), new compound **3** (t_R = 33.0 min, 3.0 mg), 8-methylpinocembrin (**12**) (t_R = 42.0 min, 8.5 mg), and 5,7-dihydroxy-6,8-dimethylflavanone (**13**) (t_R = 56.0 min, 15.5 mg) were produced from F5.3.3 (170 mg). Finally, new compound **4** (t_R = 29.0 min, 6.5 mg) and 2,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**14**) (t_R = 37.5 min, 9.5 mg) were purified by HPLC (0–45 min: 65% MeOH, 50 min: 100% MeOH) from subfraction F5.3.4 (110 mg).

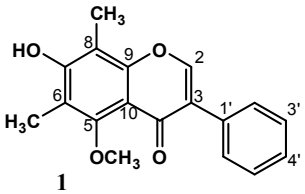
7-hydroxy-5-methoxy-6,8-dimethylisoflavone (**1**): Yellow amorphous powder; UV (MeOH) λ_{max} nm (log ε) 255 (4.32), 298 (3.79); IR (KBr) ν_{max} 3386 (OH), 2926, 1637 (C=O), 1591, 1447, 1230, 1136 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 1**; EIMS *m/z* (rel. int.): 296 ($[M]^+$, 100), 295 (31), 281 (89), 278 (57), 265 (22), 250 (17), 195 (18), 77 (19); HREIMS *m/z* 296.1047 $[M]^+$ (Calcd for $C_{18}H_{16}O_4$, 296.1049).

5,7-dihydroxy-6,8-dimethyldihydroflavonol (**2**): Brown amorphous powder; $[\alpha]_D^{25}$ –24.0° (*c* 0.08, MeOH); UV (MeOH) λ_{max} nm (log ε) 297 (4.45), 340 (3.84); IR (KBr) ν_{max} 3423 (OH), 2927, 1639 (C=O), 1469, 1282, 1123 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 1**; EIMS *m/z* (rel. int.): 300 ($[M]^+$, 67), 271 (14), 181 (100), 152 (49), 77 (10); HREIMS *m/z* 300.0999 $[M]^+$ (Calcd for $C_{17}H_{16}O_5$, 300.0998).

2,7-dihydroxy-5-methoxy-6,8-dimethylflavanone (**3**): Brown amorphous powder; UV

(MeOH) λ_{max} nm (log ε) 294 (4.47), 338 (3.87); IR (KBr) ν_{max} 3391 (OH), 2926, 1681 (C=O), 1621, 1410, 1338, 1114 cm^{-1}; ^{1}H and ^{13}C NMR data, see **Table 1**; EIMS *m/z* (rel. int.): 314 ($[M]^+$, 24), 223 (77), 195 (100), 152 (17), 77 (8); HREIMS *m/z* 314.1152 $[M]^+$ (Calcd for $C_{18}H_{18}O_5$, 314.1154).

4,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4**): Yellow amorphous powder; UV (MeOH) λ_{max} nm (log ε) 298 (3.91), 362 (4.47); IR (KBr) ν_{max} 3385 (OH), 2931, 1605 (C=O), 1545, 1437, 1229, 1164 cm^{-1}; ^{1}H and ^{13}C NMR data, see **Table 1**; EIMS *m/z* (rel. int.): 314 ($[M]^+$, 78), 313 (18), 221 (10), 194 (100), 166 (19), 136 (20); HREIMS *m/z* 314.1156 $[M]^+$ (Calcd for $C_{18}H_{18}O_5$, 314.1154).



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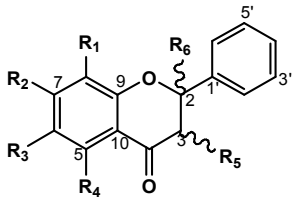

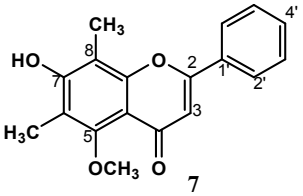

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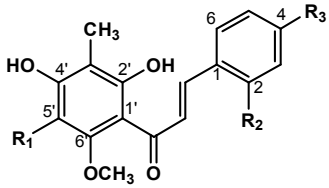


4 $R_1 = CH_3$ $R_2 = H$ $R_3 = OH$

5 $R_1 = CH_3$ $R_2 = R_3 = H$

8 $R_1 = R_2 = R_3 = H$

14 $R_1 = CH_3$ $R_2 = OH$ $R_3 = H$

2 $R_1 = R_3 = CH_3$ $R_2 = R_4 = R_5 = OH$ $R_6 = H$

3 $R_1 = R_3 = CH_3$ $R_2 = R_6 = OH$ $R_4 = OCH_3$ $R_5 = H$

6 $R_1 = R_3 = CH_3$ $R_2 = OCH_3$ $R_4 = OH$ $R_5 = R_6 = H$

9 $R_1 = CH_3$ $R_2 = OCH_3$ $R_3 = CHO$ $R_4 = OH$ $R_5 = R_6 = H$

10 $R_1 = CHO$ $R_2 = OCH_3$ $R_3 = CH_3$ $R_4 = OH$ $R_5 = R_6 = H$

11 $R_1 = CH_3$ $R_2 = OH$ $R_3 = H$ $R_4 = OCH_3$ $R_5 = R_6 = H$

12 $R_1 = CH_3$ $R_2 = OH$ $R_3 = H$ $R_4 = OH$ $R_5 = R_6 = H$

13 $R_1 = R_3 = CH_3$ $R_2 = R_4 = OH$ $R_5 = R_6 = H$

Figure 2. Chemical structures of isolated compounds

2.2.2 Cloning of novel H_1N_1 influenza NA

A full length cDNA encoding the neuraminidase of novel H_1N_1 influenza (A/California/08/2009(H_1N_1)) 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 (H_9N_2) and A/Sw/Kor/CAH1/04(H_1N_1, 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 ^oC 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 ^oC. 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 (H_1N_1 and H_9N_2) neuraminidase inhibition assay

The enzyme assay was performed as previously reported with a slight modification (*20*). In general, large-scale influenza virus suspension was prepared from MDCK cells infected with the influenza viruses, H_1N_1 and H_9N_2. The virus suspensions were treated with formaldehyde at a final concentration of 0.01% at 37^{o}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^{o}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^{o}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.

$$\% \text{ Inhibition} = \frac{100}{1 + (IC50/[I])}$$

2.2.5 Novel H_1N_1 (WT) and oseltamivir-resistant novel H_1N_1 (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.

2.2.7 Cytotoxicity assay

The MDCK cells were grown in 96-well plates at 1×10^5 cells/well for 24 hrs. The plates were replaced with media containing the serially diluted compounds. After 48 hrs incubation, a 10 μL Ez-cytox solution (Daeil lab service Co.) was added to each well. The cells were incubated further for 3 hrs at 37°C, and the optical density (OD) of the wells was determined at a test wavelength of 450 nm. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis.

2.2.8 Immunofluorescence assay (IFA)

The MDCK cells were grown on 8-well chamber slides (LAB-TEK, NUNC, USA). After incubation for 24 hrs, the media was removed and 1mL infection media (DMEM + 0.5% BSA, 1.5 μg/mL Trypsin, 1% P/S) was added into each well. The cell monolayers were infected with influenza virus (A/PR/8/34) at 1000 $TCID_{50}$/mL for 2 hrs. The solution was removed and replaced with the culture media (DMEM, 1% A/A, 5% FBS, and 2 mL L-glutamine), and treated with the compounds at the corresponding concentration. The cultures were incubated for 24 hrs at 37°C under a 5 % CO_2 atmosphere. The cells were rinsed carefully three times with PBS (pH 7.4) and fixed with a 4% paraformaldehyde solution for 30 min at room temperature. After washing twice with PBS (pH 7.4), 1 mL of 0.2% triton X-100 was added to the cells. After blocking with 1 mL 1% BSA for 1 hr, the cells were incubated with monoclonal antibodies against the influenza virus M1 protein (Santa Cruz, USA). After washing with PBS (pH 7.4), the cells were incubated with the secondary FITC-conjugated goat

anti-mouse IgG antibody (Jackson ImmunoResearch, Inc.) for 1 hr. After washing three times with PBS (pH 7.4), the cells were stained with a 500 μL DAPI solution for 1 min at RT. After washing with PBS, the slides were mounted with a mounting medium for fluorescence (VECTASHIELD, Vector Laboratories, Inc.) and observed by fluorescence microscopy (Carl Zeizz, Germany).

3. Results and Discussion

3.1 Structure determination of C-methylated Flavonoids from *Cleistocalyx operculatus*

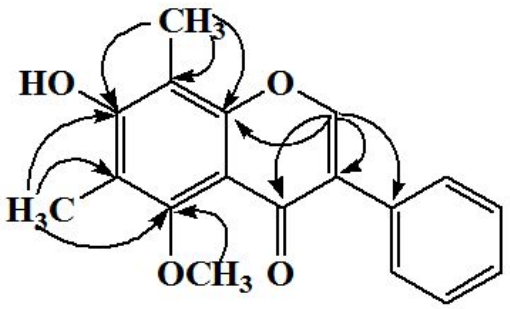
A succession of chromatographic procedures (silica gel, Shephadex LH-20, RP-18, and HPLC) of the methanol extract of *C. operculatus* afforded fourteen compounds, including four new (**1**–**4**) along with ten known (**5**–**14**) C-methylated flavonoid derivatives as active principles.

Compound **1** obtained as a yellow amorphous powder was determined to have a molecular formula of $C_{18}H_{16}O_4$ based on HREIMS ($[M]^+$, *m/z* 296.1047). The IR spectrum revealed absorption bands at 3386 and 1637 cm^{-1}, which were consistent with the presence of a hydroxy group and a conjugated carbonyl group. The UV absorbance at 255 nm and a ^1H singlet resonance at δ_H 7.88 and corresponding olefinic oxymethine signal at δ_C 151.0 showed it to be an isoflavone derivative (*21*). The ^1H NMR spectrum (**Table 1**) showed three aromatic protons and two aromatic protons multiplets at δ_H 7.34 and 7.51, respectively, which are typical for an isoflavone with an unsubstituted B ring. In addition, the ^1H and ^{13}C NMR spectra of **1** indicated the presence of a methoxy group [δ_H 3.81 (3H, s, OCH_3-5)] and two methyl groups [δ_H 2.23 (3H, s, CH_3-6) and 2.28 (3H, s, CH_3-8)]. The position of the two methyl groups at C-6 and C-8 on the A ring was established through HMBC correlations (CH_3-6/C-5, C-6, C-7, and CH_3-8/C-7, C-8, C-9, respectively) (**Figure 3**). Further support for these assignments was obtained by a comparison with the ^1H and ^{13}C NMR spectroscopic data of 4′,5,7-trihydroxy-6,8-dimethylisoflavone (*24*). The HMBC correlation between the methoxy protons (δ_H 3.81) and C-5 (δ_C 156.3) revealed a methoxy group at C-5. Finally, the hydroxy group was determined to be attached to C-7 by the HMBC correlations between the methyl protons of CH_3-6 (δ_H 2.23), CH_3-8 (δ_H 2.28) and C-7 (δ_C 156.7) (**Figure 3**). Therefore, the structure of **1** was determined to be 7-hydroxy-5-methoxy-6,8-dimethylisoflavone.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for 1–4 and 7.

Position	1[a]		2[b]		3[a]		4[c]		7[a]	
	δ_H mult. (*J* in	δ_C	δ_H mult. (*J* in	δ_C	δ_H mult. (*J* in	δ_C	δ_H mult. (*J* in	δ_C	δ_H mult. (*J* in	δ_C
1								128.		
2	7.88 s	151.	5.04 d (11.0)	85.0		103.8	7.65 d (8.5)	131.		160.9
3		125.	4.51 d (11.0)	74.1	3.16 d (13.5)	42.0	6.94 d (8.5)	116.	6.71 s	108.2
					3.24 d (13.5)					
4								160.		
5		156.		158.9		155.2	6.94 d (8.5)	116.		155.8
6		115.		104.5		109.3	7.65 d (8.5)	131.		115.3
7		156.		164.7		162.3				156.9
8		106.		105.5		101.6				107.2
9		154.		160.3		167.9				154.9
10		113.		101.8		104.8				112.3
α							7.92 d (15.5)	124.		
β							7.82 d (15.5)	144.		
1'		132.		139.1		132.9		109.		131.9
2'	7.51 d (8.4)	129.	7.56 d (8.0)	129.0	7.29 d (8.0)	130.6		162.	7.90 m	126.0
3'	7.34 m	128.	7.41 m	129.6	7.25 m	128.3		107.	7.52 m	129.0
4'	7.34 m	127.	7.41 m	129.9	7.23 m	127.4		161.	7.52 m	131.2
5'	7.34 m	128.	7.41 m	129.6	7.25 m	128.3		110.	7.52 m	129.0
6'	7.51 d (8.4)	129.	7.56 d (8.0)	129.0	7.29 d (8.0)	130.6		159.	7.90 m	126.0
CH_3-6, 3'	2.23 s	8.2	1.97 s	7.6	2.03 s	7.2	2.09 s	8.3	2.26 s	8.3
CH_3-8, 5'	2.28 s	8.1	2.02 s	8.1	2.09 s	7.9	2.15 s	9.0	2.44 s	8.5
OH-2'							13.96 s			
OCH_3-5,	3.81 s	61.7			3.97 s	61.7	3.69 s	62.6	3.87 s	61.8
CO		175.		198.9		193.7		193.		177.8

[a] Recorded in $CDCl_3$. [b] Recorded in CD_3OD, [c] Recorded in acetone-d_6.



1

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

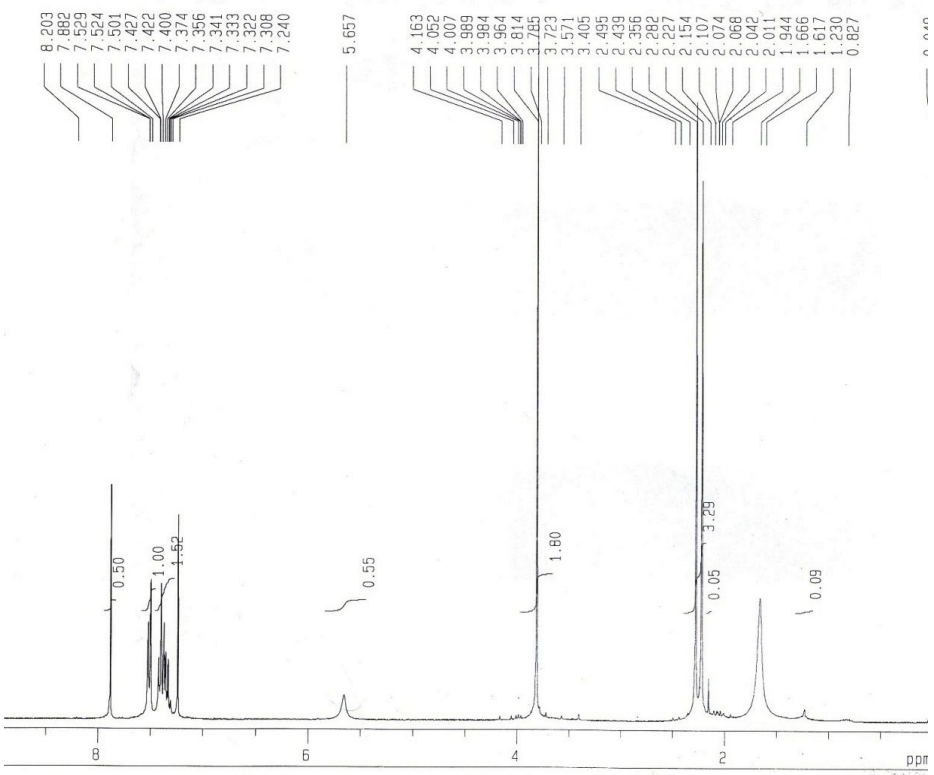


Figure 4. ^{1}H NMR (500 MHz, $CDCl_3$) of new compound **1**

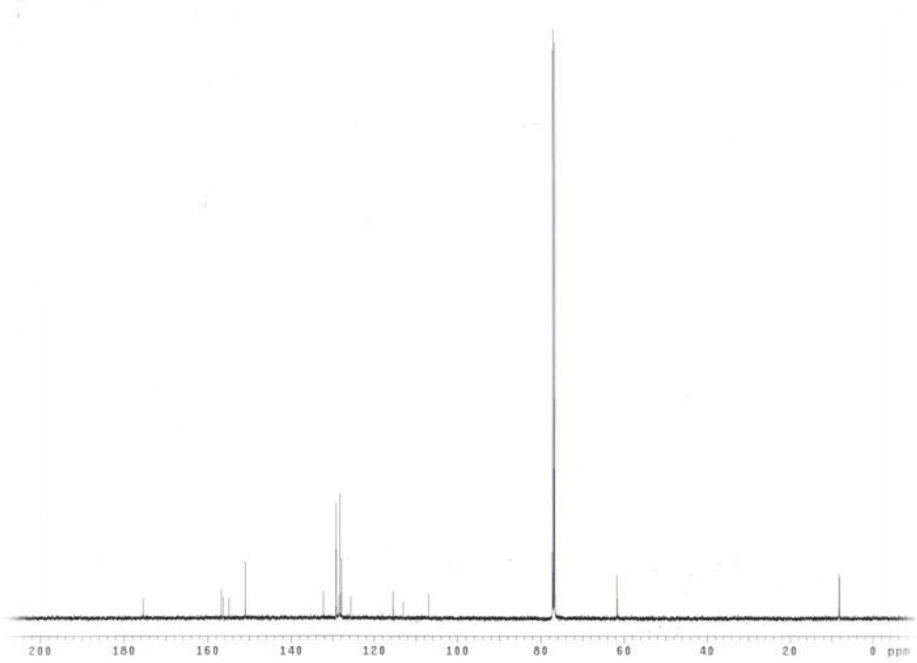


Figure 5. ^{13}C NMR (125 MHz, $CDCl_3$) of new compound **1**

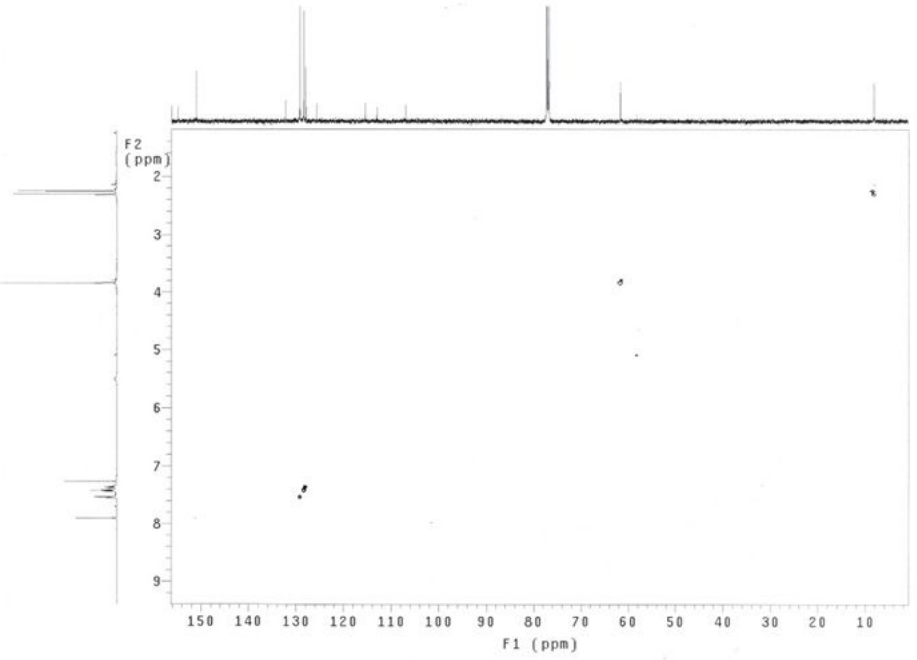


Figure 6. HSQC spectrum of new compound **1**

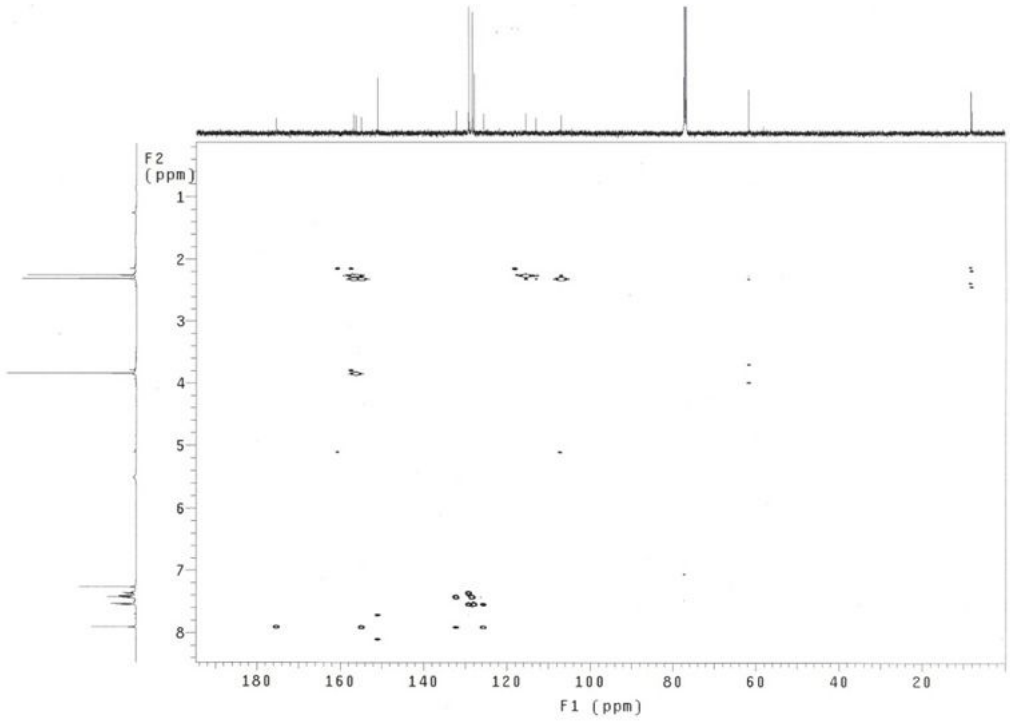


Figure 7. HMBC spectrum of new compound **1**

Compound **2**, a brown amorphous powder, was determined to be $C_{17}H_{16}O_5$ based on HREIMS ($[M]^+$, *m/z* 300.0999). The IR absorption bands at 3423 and 1639 cm^{-1} indicated the presence of hydroxy and conjugated carbonyl groups, respectively. The UV spectrum showed the characteristic absorbance bands for a dihydroflavonol (297 and 340 nm) (*19*). The ^1H NMR contained signals for two AB systems at δ_H 4.51 (1H, d, J = 11.0 Hz) and 5.04 (1H, d, J = 11.0 Hz) that were characteristic of H-2 and H-3 in the axial conformation of a 2,3-*trans*-dihydroflavonol (*23*), and 7.41 (3H, m) and δ_H 7.56 (2H, m), which is indicative of an unsubstituted B ring of the flavonoid, and the signals for two methyl groups [δ_H 1.97 (3H, s, CH_3-6) and 2.02 (3H, s, CH_3-8)]. Consistent with the above ^1H NMR analysis, the ^{13}C NMR spectrum of this compound showed the signals for all 17 carbons. Fifteen signals were accounted for by the flavonoid skeleton. Among them, three signals belonged to aromatic

carbons bearing an oxygen atom [δ_C 158.9 (C-5), 160.3 (C-9) and 164.7 (C-7)]. A comparison of this data with that of demethoxymatteucinol (*24*), indicated compound **2** to be a dihydroflavonol with two hydroxy and two methyl substituents on the A ring. The positions of both methyl groups at C-6 and C-8 were further confirmed by HMBC correlations (CH_3-6/C-5, C-6, C-7, and CH_3-8/C-7, C-8, C-9, respectively) (**Figure 8**). Compound **2** was identified to be 5,7-dihydroxy-6,8-dimethyldihydroflavonol.

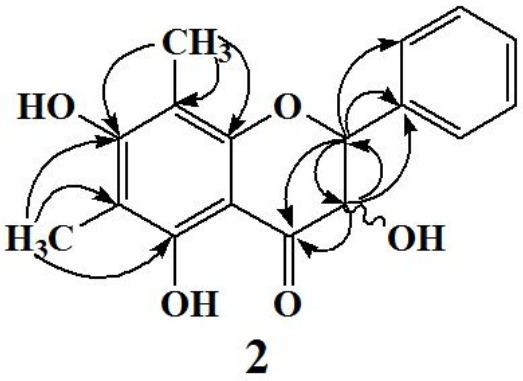


Figure 8. Key HMBC (H → C) correlations for new compound **2**

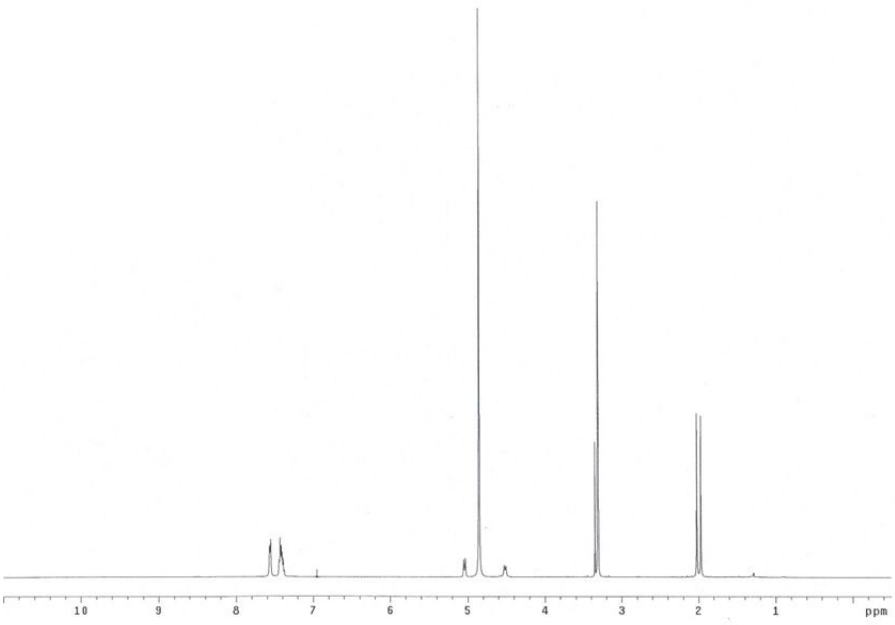


Figure 9. ^{1}H NMR (500 MHz, CD_3OD) of new compound **2**

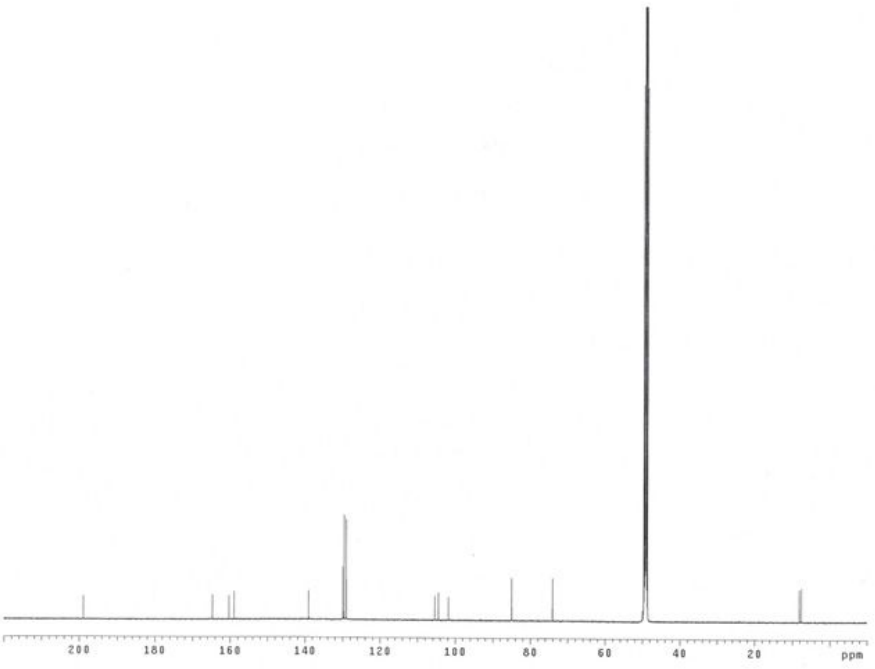


Figure 10. ^{13}C NMR (125 MHz, CD_3OD) of new compound **2**

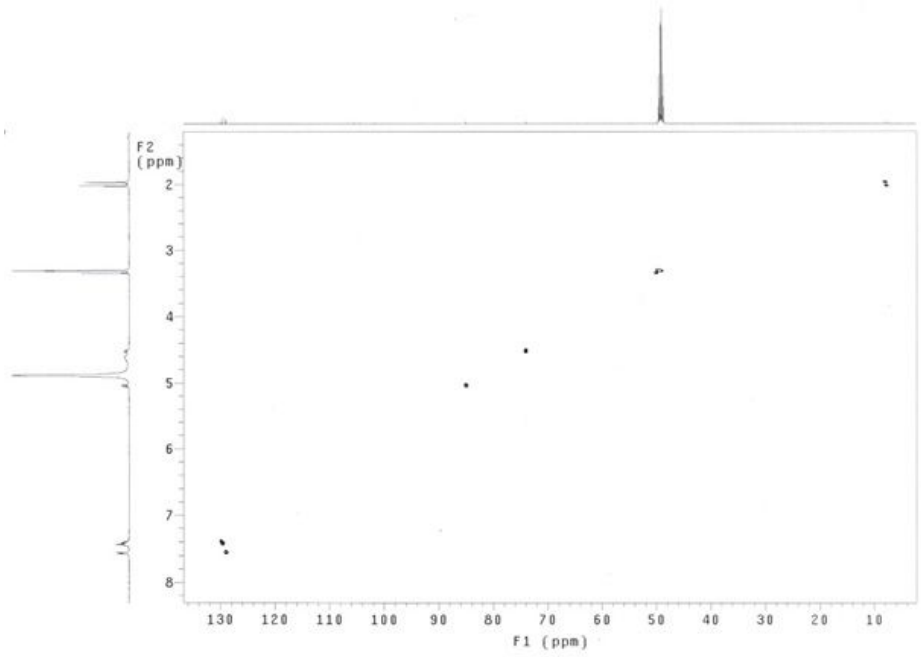


Figure 11. HSQC spectrum of new compound **2**

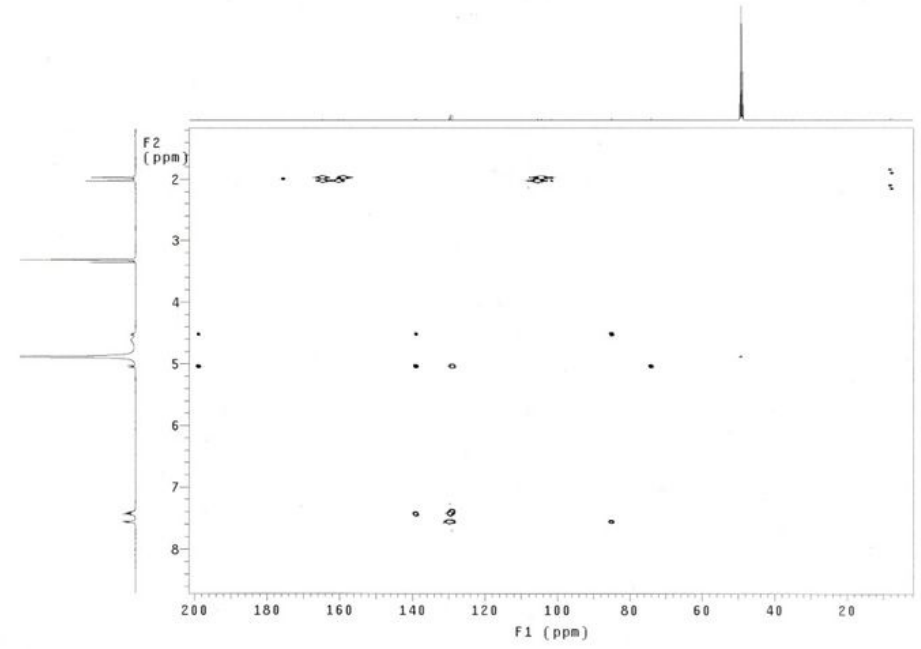
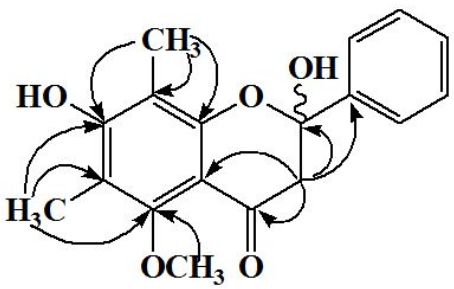


Figure 12. HMBC spectrum of new compound **2**

Compound **3** was obtained as a brown amorphous powder and shown to have a molecular formula of $C_{18}H_{18}O_5$ from its HREIMS ($[M]^+$, *m/z* 314.1152). The UV spectrum showed strong absorption at 294 nm and a shoulder at 338 nm, which is typical of flavanones (*21*). The IR spectrum indicated hydroxy (3391 cm^{-1}) and carbonyl (1681 cm^{-1}) functionality. The ^1H NMR spectrum showed two AB protons [δ_H 3.16 (1H, d, *J* = 15.0 Hz, H-3ax) and 3.24 (1H, d, *J* = 15.0 Hz, H-3eq)], and signals for a methoxy group [δ_H 3.97 (3H, s, OCH_3-5)], and two methyl groups [δ_H 2.03 (3H, s, CH_3-6) and 2.09 (3H, s, CH_3-8)]. The absence of a double doublet at δ_H 5.20, indicated the absence of an H-2 proton, which normally couples with the H-3 protons in flavanones (*21*). Substitution with a hydroxy group was supported by the presence of two AB protons (H-3) and an oxygenated sp^3 carbon signal at δ_C 103.8 (C-2) in the ^{13}C NMR spectrum (*25*). The coupled three- and two-proton multiplets at δ_H 7.24 and 7.29, respectively, indicated that **3** has an unsubstituted B ring (*16*). Finally, the substitution pattern on the A ring was determined to be 7-hydroxy-5-methoxy-6,8-dimethyl from the ^1H–^{13}C HSQC and HMBC correlations (CH_3-6/C-5, C-6, C-7; CH_3-8/C-7, C-8, C-9; and OCH_3-5/C-5) (**Figure 13**). Therefore, the structure of **3** was determined to be 2,7-dihydroxy-5-methoxy-6,8-dimethylflavanone.



3

Figure 13. Key HMBC (H → C) correlations for new compound **3**

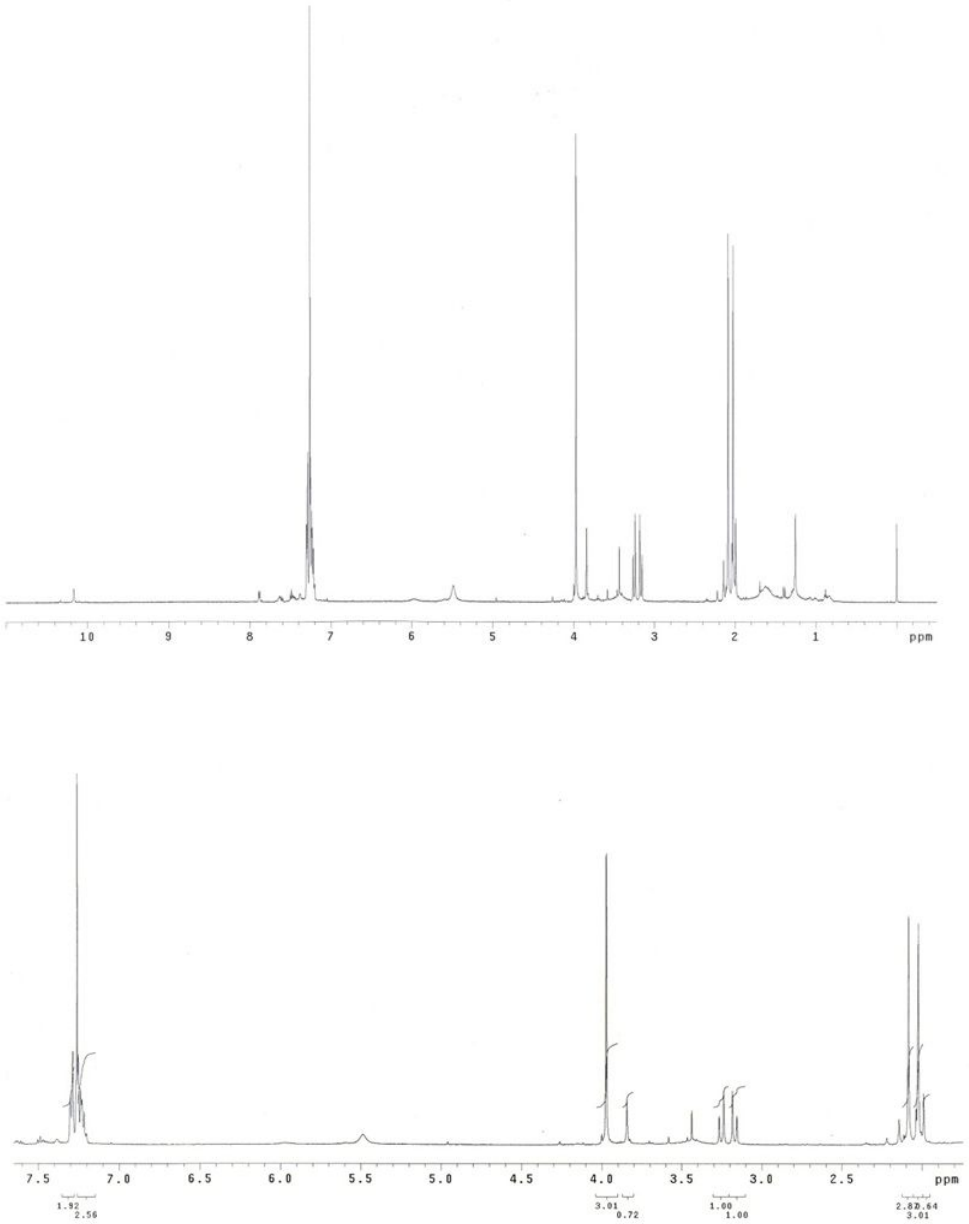


Figure 14. ^{1}H NMR (500 MHz, $CDCl_3$) of new compound **3**

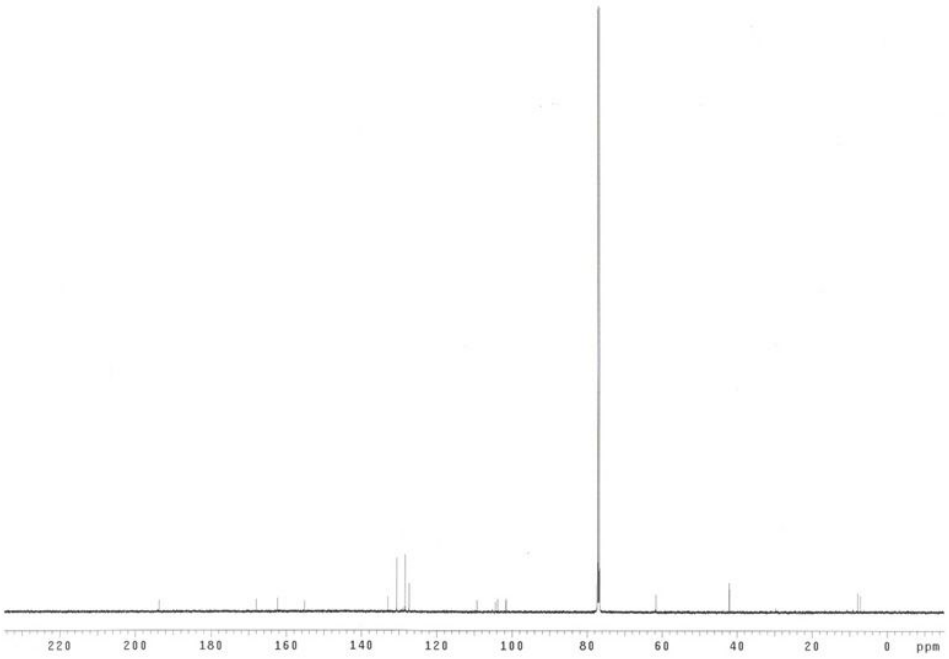


Figure 15. ^{13}C NMR (125 MHz, $CDCl_3$) of new compound **3**

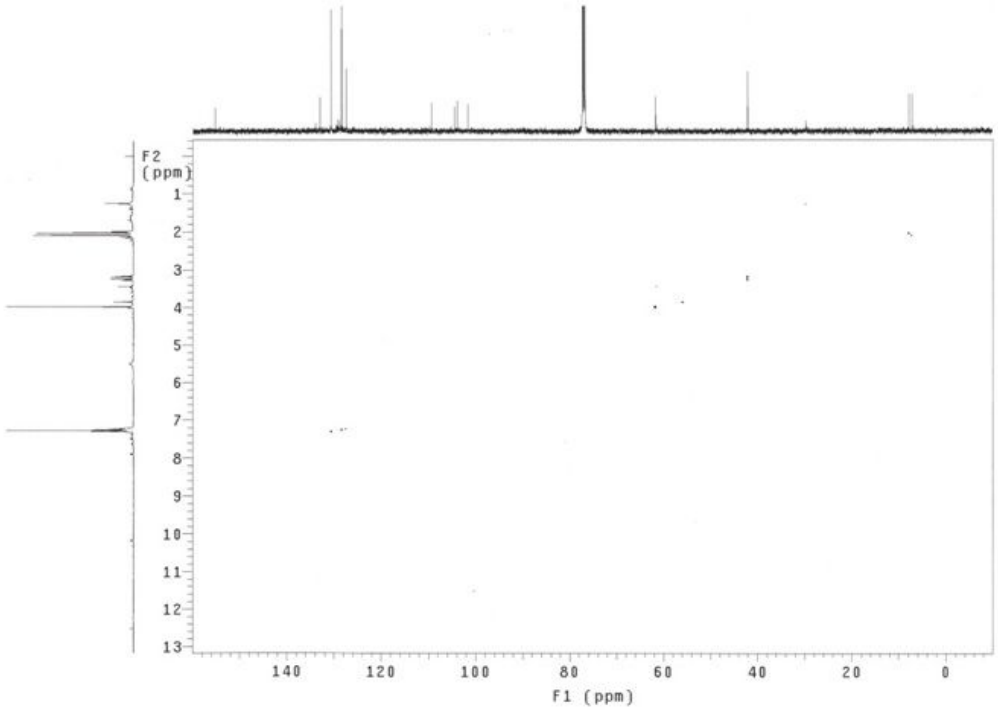


Figure 16. HSQC spectrum of new compound **3**

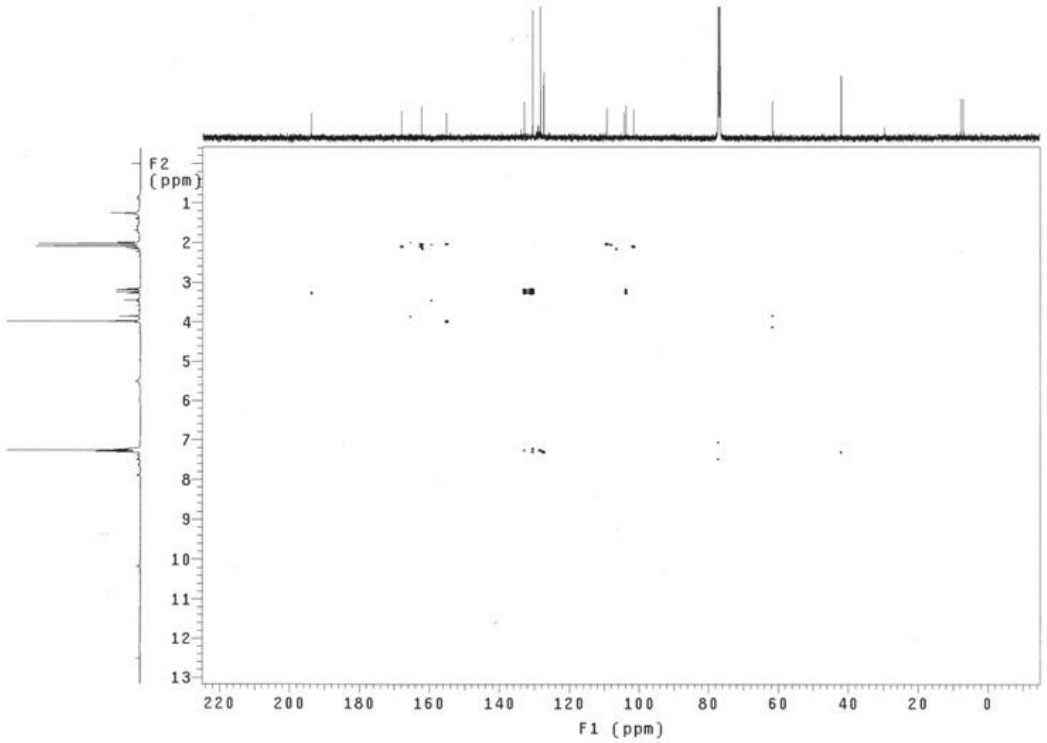
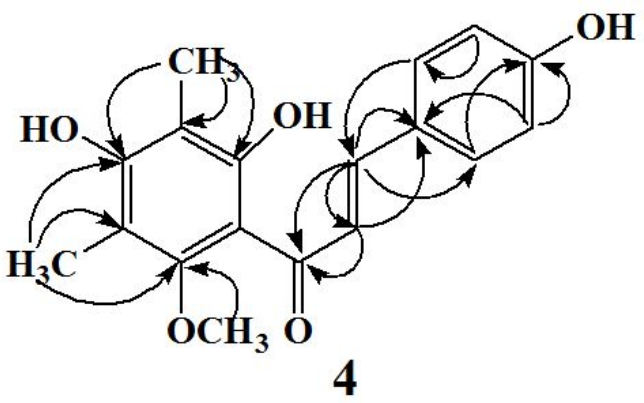


Figure 17. HMBC spectrum of new compound **3**

Compound **4** was obtained as a yellow amorphous powder. Its IR spectrum revealed absorption bands at 3385 cm^{-1} for one or more hydroxy groups and 1605 cm^{-1}. The ^{1}H NMR spectrum of **4** showed signals for a methoxy group δ_H 3.69 (3H, s, OCH_3-6′), two *trans*-olefinic protons [δ_H 7.82 (1H, d, J = 15.5 Hz, H-β) and 7.92 (1H, d, J = 15.5 Hz, H-α)], two methyl groups [δ_H 2.09 (3H, s, CH_3-3′) and 2.15 (3H, s, CH_3-5′)], and an A_2B_2 system of aromatic protons [δ_H 6.94 (2H, d, J = 8.5 Hz, H-3, 5) and 7.65 (2H, d, J = 8.5 Hz, H-2, 6)]. Consistent with the above ^{1}H NMR analysis, the ^{13}C NMR spectrum displayed signals corresponding to the methoxy group δ_C 62.6 (OCH_3-6′), two olefinic carbons [δ_C 124.4 (C-α), 144.3 (C-β)], two methyl groups [δ_C 8.3 (CH_3-3′), and 9.0 (CH_3-5′)], a conjugated ketone δ_C 193.8, and twelve carbons of the two aromatic rings. The 1D NMR spectroscopic data of compound **4** was similar

to that of 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone, except for an additional hydroxy group attached to the A ring from the aromatic A_2B_2 proton system (*26*). This was further supported by the molecular ion peak at *m/z* 314.1156 $[M]^+$ in the HREIMS, which indicated a molecular formula of $C_{18}H_{18}O_5$. The position of the two methyl groups at C-3′ and C-5′ was established through HMBC correlations (CH_3-3′/C-2′, C-3′, C-4′, and CH_3-5′/C-4′, C-5′, C-6′, respectively). The HMBC correlation between the methoxy singlet at δ_H 3.69 and the C-6′ peak at δ_C 159.8, which was in turn correlated with the 5′-methyl protons at δ_H 2.15, indicated the methoxy group to be located on the B ring. Finally, the additional hydroxy group was found to be attached to C-4 according to the observation of an aromatic A_2B_2 proton system (δ_H 6.94 and 7.65) and their HMBC correlations (**Figure 18**). Therefore, compound **4** was determined to be 4,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone.



4

Figure 18. Key HMBC (H → C) correlations for new compound **4**

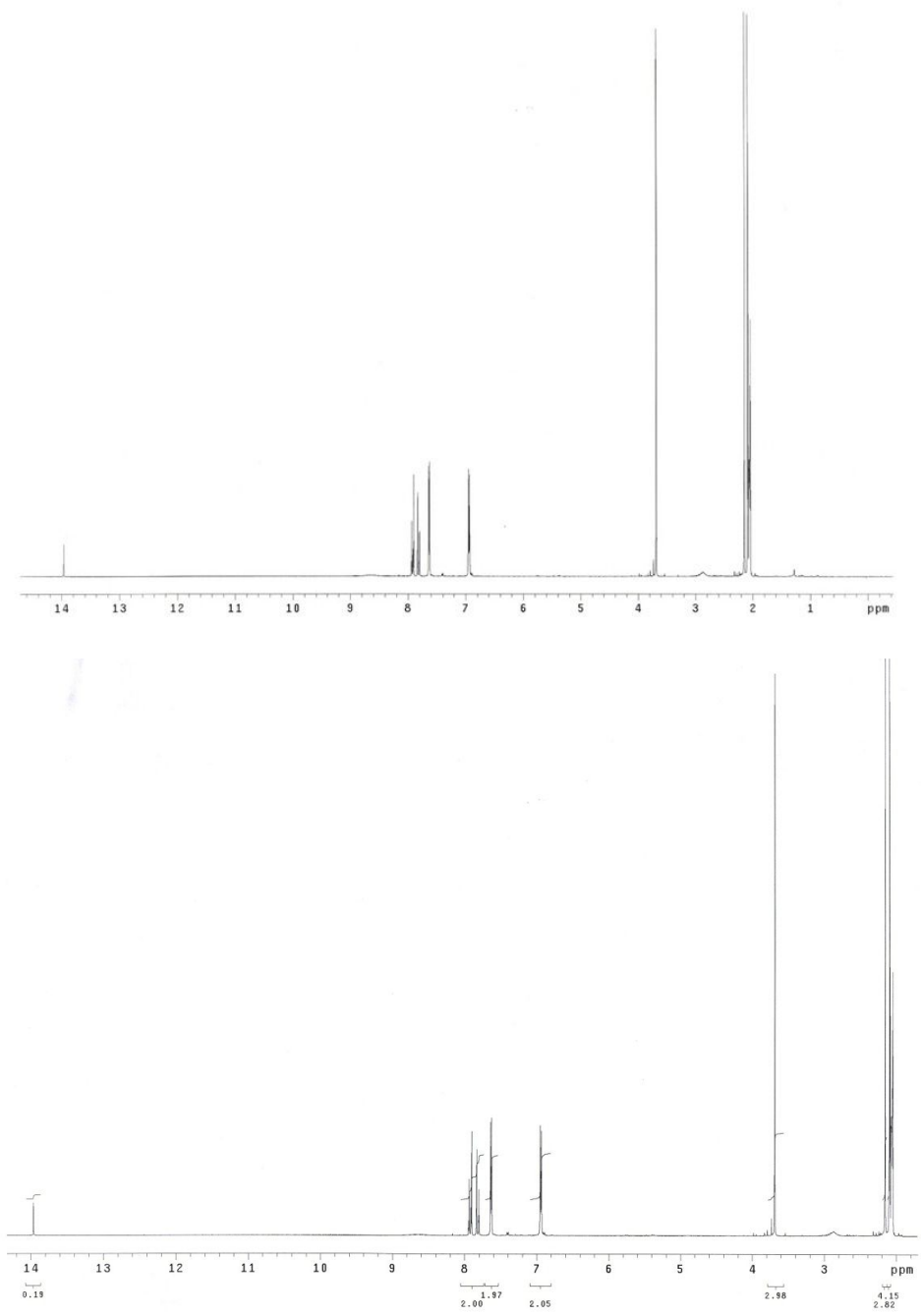


Figure 19. ^{1}H NMR (500 MHz, CD_3COCD_3) of new compound **4**

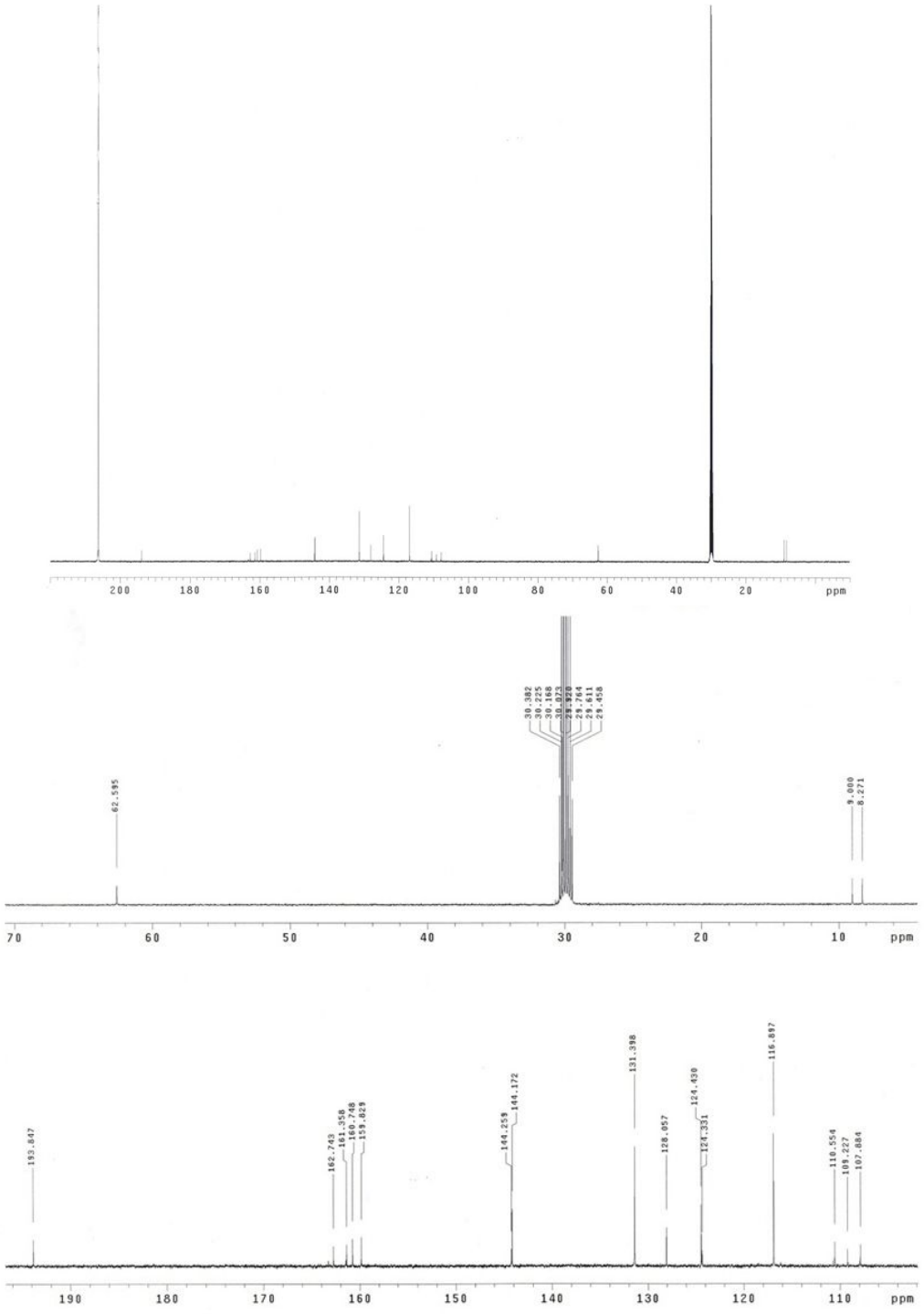


Figure 20. ^{13}C NMR (125 MHz, CD_3COCD_3) of new compound **4**

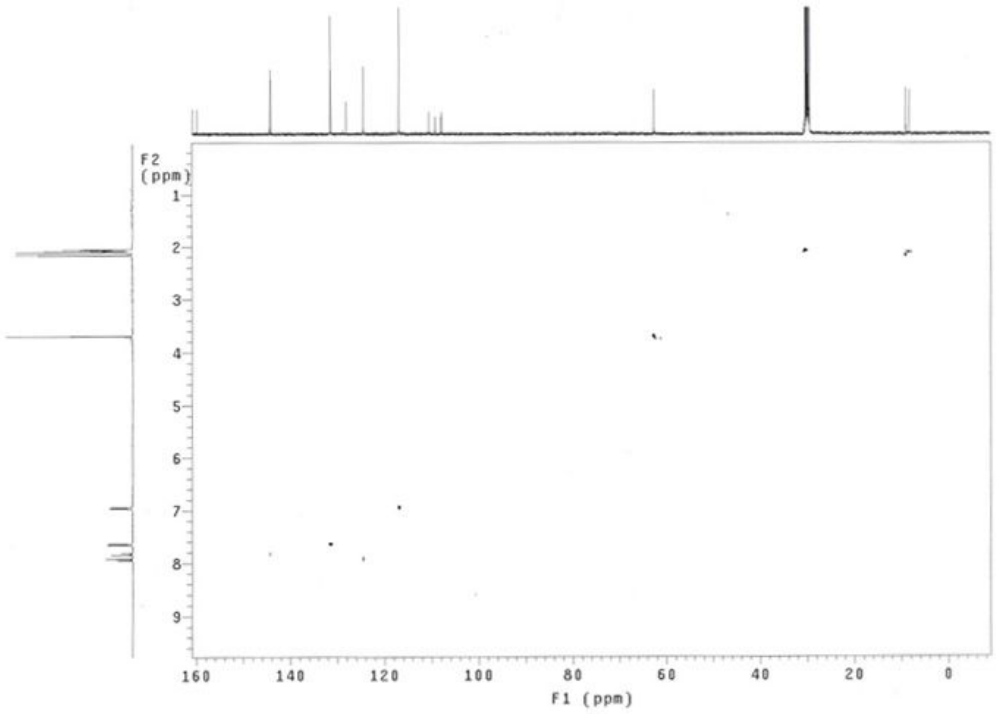


Figure 21. HSQC spectrum of new compound **4**

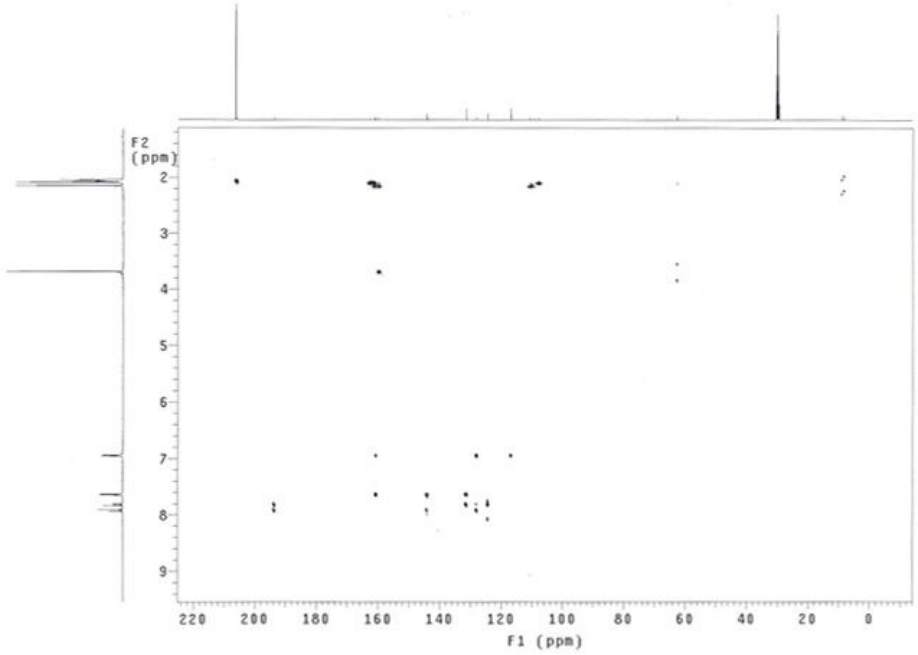


Figure 22. HMBC spectrum of new compound **4**

Compound **7** was determined to be 7-hydroxy-5-methoxy-6,8-dimethylflavone. This compound has been reported as a synthetic product but only limited spectroscopic data has been reported (*27*) (**Table 1**). The structure of known compounds **5**, **6**, and **8**–**14** were identified to be 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**5**) (*26*), 5-hydroxy-7-methoxy-6,8-dimethylflavanone (**6**) (*28*), 2′,4′-dihydroxy-3′-methyl-6′-methoxychalcone (**8**) (*29*), 6-formyl-8-methyl-7-*O*-methylpinocembrin (**9**) (*30*), (2*S*)-8-formyl-5-hydroxy-7-methoxy-6-methylflavanone (**10**) (*5*), 7-hydroxy-5-methoxy-8-methylflavanone (**11**) (*31*), 8-methylpinocembrin (**12**) (*32*), 5,7-dihydroxy-6,8-dimethylfavanone (**13**) (*29*) and 2,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**14**) (*26*) by a comparison of their physicochemical and spectroscopic data with those reported in the literature. To our knowledge, this is the first report of the seven known compounds (**6**–**9**, **11**, **12**, and **14**) being isolated from *C. operculatus*.

3.2 Effects of the isolated compounds on NAs from two influenza viral strains, H_1N_1 and H_9N_2

All isolates were examined for their inhibitory activity against swine influenza virus (H_1N_1) NA using oseltamivir phosphate (Hoffman-La Roche Ltd, Basel, Switzerland) as a positive control (*33*). All compounds, with the exception of compounds **6**, **11**, and **12** inhibited NA in a dose-dependent manner. The chalcone derivatives (**4**, **5**, **8**, and **14**) exhibited stronger activity than others, flavanone, flavonol or isoflavone skeletons (**Table 2**). Furthermore, compound **4** (IC_{50} 6.42 ± 0.43 μg/mL), a C-4 hydroxy derivative, exhibited greater activity against NA than the other chalcones **5** and **14**. Similarly, the presence of a C-3 (C-6) methyl group might increase the NA inhibitory properties, as observed in compound **5** (**13**), compared to the structurally similar compound **8** (**12**). Interestingly, the inhibitory effects on the avian

influenza virus (H_9N_2) neuraminidase of these isolated compounds also revealed IC_{50} values in the same order of magnitude against NA from the swine influenza virus (H_1N_1) (**Table 2** and **Figure 23**).

Table 2. Inhibitory effects of compounds **1**–**14** on neuraminidase activity

Compound	H_1N_1 IC_{50} (μg/mL)[a]	H_9N_2 IC_{50} (μg/mL)[a]	H_1N_1 (WT) IC_{50} (μg/mL)[a]	H_1N_1 (H274Y)	
				IC_{50} (μg/mL)[a]	Fold increase vs. WT
1	127.43 ± 2.42	115.74 ± 2.62	NT[c]	NT[c]	
2	112.40 ± 2.25	110.36 ± 1.97	NT[c]	NT[c]	
3	88.73 ± 2.07	89.42 ± 2.55	NT[c]	NT[c]	
4	6.42 ± 0.43	5.83 ± 0.43	2.56 ± 0.33	1.04 ± 0.42	2.46
5	9.68 ± 0.69	6.48 ± 0.70	10.50 ± 0.82	1.50 ± 0.26	7.00
6	>150	>150	NT[c]	NT[c]	
7	122.64 ± 2.77	107.41 ± 2.15	NT[c]	NT[c]	
8	24.35 ± 2.06	18.88 ± 2.33	26.63 ± 1.52	7.39 ± 0.86	3.60
9	83.80 ± 2.97	79.95 ± 1.53	NT[c]	NT[c]	
10	90.46 ± 3.15	93.26 ± 2.14	NT[c]	NT[c]	
11	>150	>150	NT[c]	NT[c]	
12	>150	>150	NT[c]	NT[c]	
13	94.74 ± 2.63	90.63 ± 2.43	NT[c]	NT[c]	
14	8.83 ± 1.12	7.26 ± 0.61	3.38 ± 0.68	0.80 ± 0.09	4.23
Oseltamivir[b]	39.04 ± 1.03 (ng/mL)	4.24 ± 0.82 (ng/mL)	21.13 ± 1.36 (ng/mL)	5.04 ± 0.41	

[a]All compounds were examined in a set of triplicated experiment.

[b]The compound was used as positive control.

[c]NT: not tested.

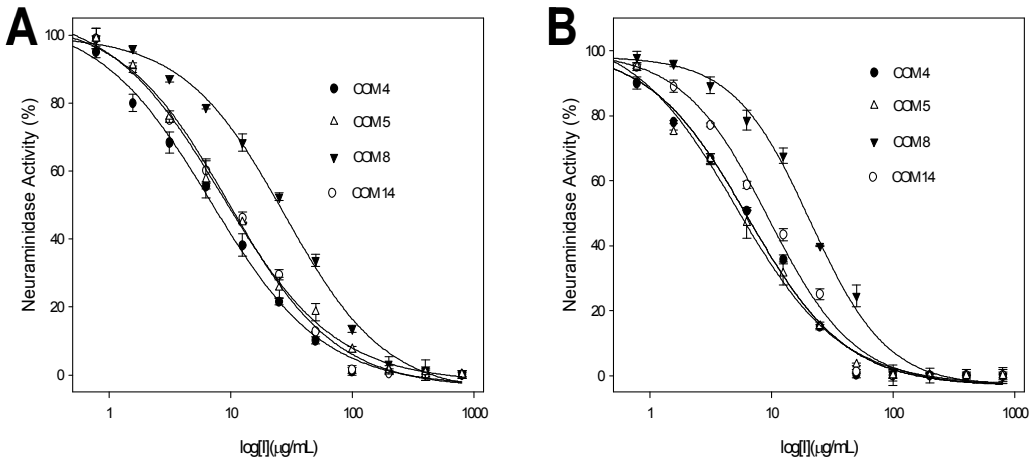


Figure 23. (A–B) Effects of compounds (4, 5, 8, and 14) on the activity of NAs from influenza A (H_1N_1 and H_9N_2) for the hydrolysis of 4-MU-NANA at 37°C. Inhibitor concentrations are displayed on logarithmic scales. The IC50 is identified from the midpoint (neuraminidase activity = 50%) of the semilog plot.

3.3 Effects of the isolated compounds on NAs from novel H_1N_1 and oseltamivir-resistant novel H_1N_1 (H274Y) expressed in 293T cells

This study examined whether the isolates are also effective in inhibiting NAs from the wild-type novel swine flu (WT) virus and oseltamivir-resistant virus with a H274Y mutation (*20*). The most active compound **4** inhibited the NA derived from the wild-type with an IC_{50} of 2.56 ± 0.33 μg/mL. Interestingly, tested compounds showed stronger activity against the H274Y mutant form than the novel H_1N_1 form (from 2.46 to 7.00-fold decreases in IC_{50} values). While oseltamivir, as the positive control, displayed excellent inhibition (IC_{50} 21.13 ± 1.36 ng/mL) of the NA from the wild-type, its inhibitory activity on oseltamivir-resistant novel influenza (H274Y) decreased dramatically to an IC_{50} of 5.04 ± 0.41 μg/mL (**Table 2**).

3.4 Inhibition pattern of the compounds on neuraminidase of H_1N_1

To study the mode of inhibition, both the double reciprocal Lineweaver-Burk and Dixon plots were used (**Figure 24**). As shown in **Figure 24A–D**, the V_{max} values decreased relative to the concentration without changing K_m for the substrate (in Lineweaver-Burk plot: slope of the line = K_m/V_{max}, x-intercept = $-1/K_m$), and the lines intersect at a value of 1/[S] less than zero on the *x*-axis (at 1/(intensity/min). This suggests that these compounds are noncompetitive inhibitors of neuraminidase. To obtain the values of the inhibition constant K*i*, a Dixon plot was used by plotting 1/(intensity/min) as a function of [Inhibitor] for each substrate concentration, and the values of –K*i* was determined from the point on the *x*-axis value where the lines intersect, as illustrated in **Figure 24E-H**. A summary of the K*i* values for the tested compounds was shown in **Table 2**.

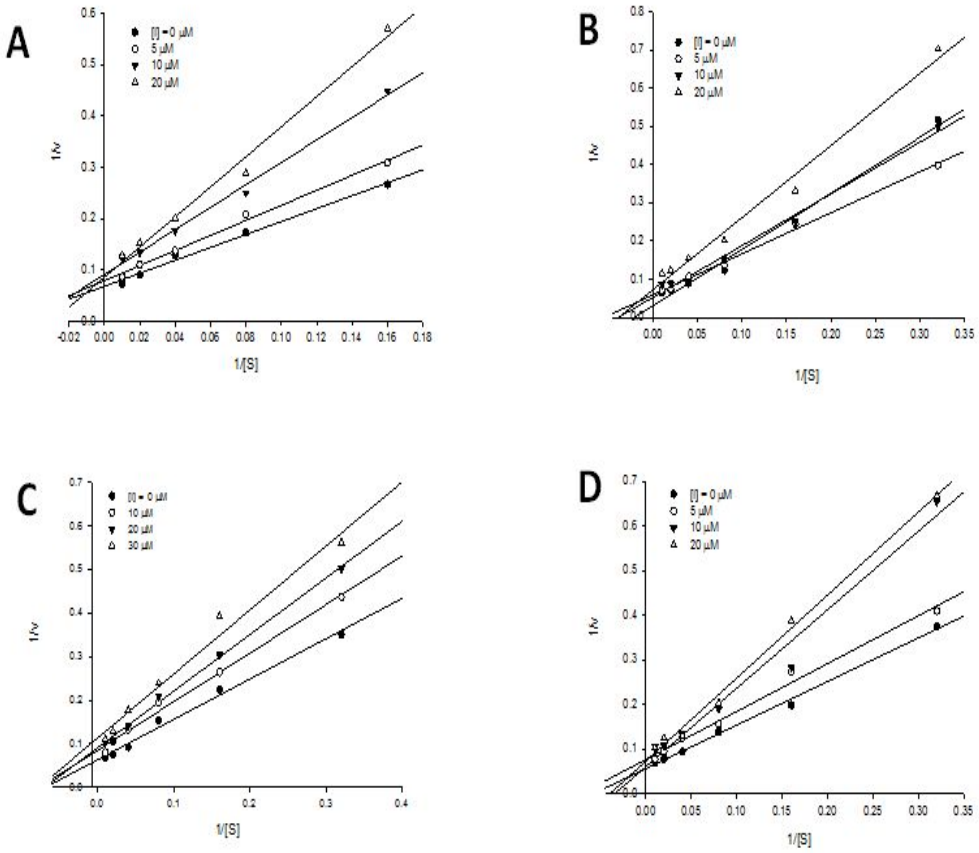


Figure 24. (**A-D**) Graphical determination of the type of inhibition for isolated compounds. Lineweaver-Burk plots for the inhibition of compounds **4**, **5**, **8**, and **14** on NA from influenza A (H_1N_1) for the hydrolysis of substrate. Data are expressed as mean reciprocal of intensity/min for n = 3 replicates at each substrate concentration.

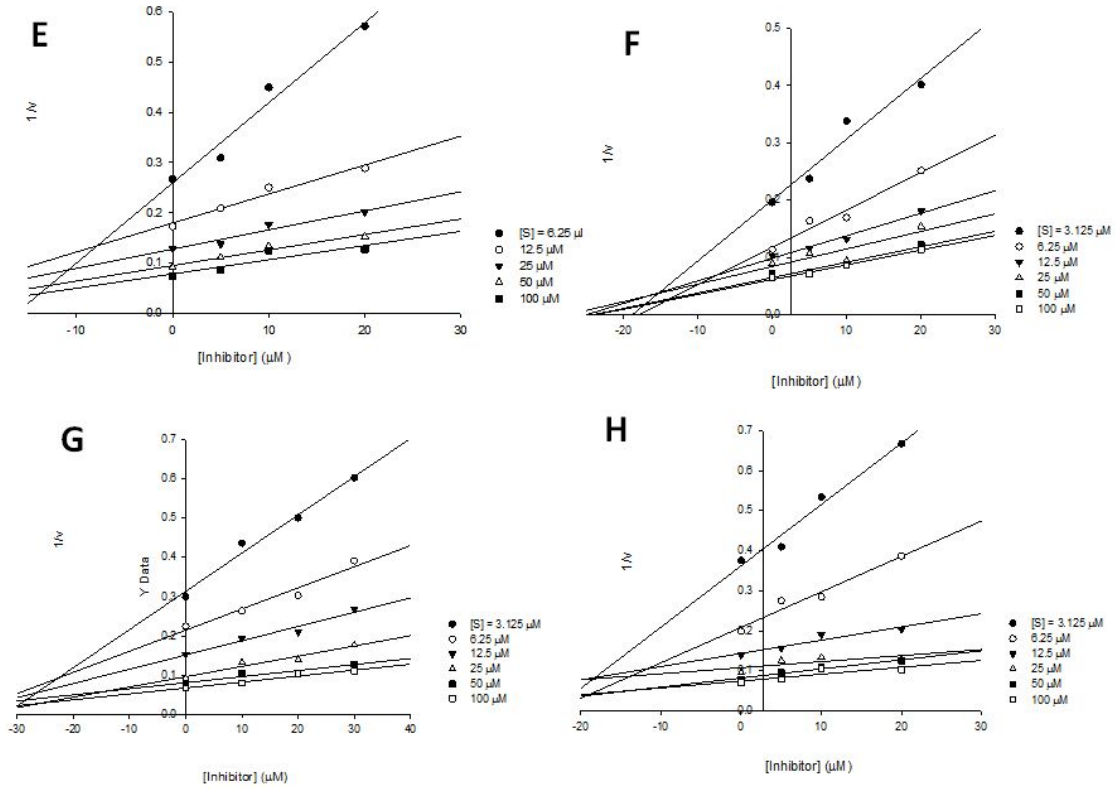


Figure 24. (E–H) Graphical determination of the type of inhibition for isolated compounds. Dixon plots for compounds **4**, **5**, **8**, and **14** determining the inhibition constant K*i*. The K*i* value is determined from the negative of the *x*-axis value at the point of intersection of the three lines. Data are expressed as mean reciprocal of intensity/min for n = 3 replicates at each substrate concentration.

3.5 Activity of compounds 4, 5, 8, and 14 against influenza A/PR/8/34 (H_1N_1) using the CPE reduction assay in MDCK cells

As shown in **Table 3**, tested compounds exhibited dose-dependent anti-influenza virus activity, with SI values ranging from 5.01 to > 25.97. Compound **4**, which was the most effective with EC_{50} values of 1.54 μg/mL and CC_{50} > 40 μg/mL, showed a high SI value > 25.97 (SI = CC_{50}/EC_{50}). This suggests that compound **4** may be a potent antiviral agent against the influenza virus with a strong protective effect and low toxicity to the host MDCK cells.

Table 3. Antiviral activities of compounds **4**, **5**, **8**, and **14** against A/PR/8/34 (H_1N_1) in MDCK cells using the CPE reduction assay.

Compound	CC_{50} (ug/mL)	EC_{50} (ug/mL)	SI
4	>40	1.54	> 25.97
5	23.36	2.30	5.01
8	38.34	2.31	16.55
14	> 40	2.76	> 14.49
Tamiflu	-	0.92	-

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

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

SI: selective index, CC_{50}/EC_{50}

(Tamiflu: % inhibition at concentration of 40 ug/mL = 7%)

3.6 Effects of compound 4 on novel influenza H_1N_1 in immunofluorescence assay

The most active compounds were then evaluated for their cytotoxicity against MDCK cells to select an active compound without cytotoxicity for an immunofluorescence experiment. Among those compounds tested in the cytotoxic assay, only one isolate, compound **5**, showed slight cytotoxicity (CC_{50}, 23.36 μg/mL) against MDCK cells. Therefore, the most active compound in influenza NAs assays, compound **4**, was used for the cytoprotective evaluation using an immunofluorescence assay. As shown in **Figure 25**, treatment of the cells with compound **4** (5 μg/mL) or oseltamivir reduced the number of fluorescence-positive cells.

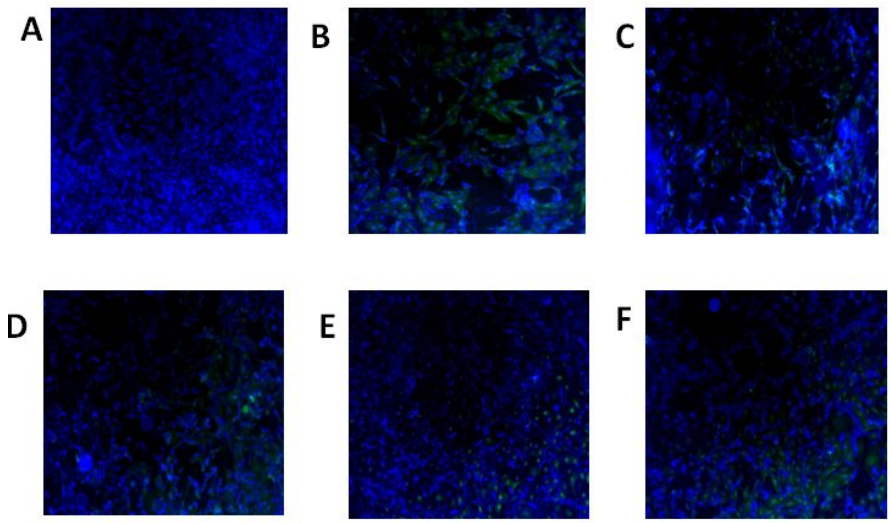


Figure 25. The antiviral effects of a representative compound **4** on influenza A/PR/8/34 in immunofluorescence assay. (**A**) Mock-inoculated MDCK cells show no positive fluorescence reaction in the cells. (**B**) Virus-infected MDCK cells without compound **4**. There are many positive cells (green). (**C-D**) Virus-infected MDCK cells with oseltamivir (2 and 0.5 μg/mL). (**E-F**) Virus-infected MDCK cells with compound **4** (5 and 1 μg/mL). There are remarkably reduced positive cells.

4. Conclusion

The emergence of drug-resistant influenza viruses and the threat of pandemics highlight the need for novel and effective antiviral agents (*34*). 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 *C.opercutus* buds with the target enzyme NA from two influenza viral strains (H1N1 and H9N2), novel H1N1 and oseltamivir-resistant novel H1N1. Four new (**1**–**4**) and ten known (**5**−**14**) C-methylated flavonoids were isolated from a methanol extract of *C.operculatus* buds and shown to be effective inhibitors of neuraminidase from various H_1N_1 strains. Data showed that four out of the fourteen compounds exhibited significant inhibitory effects against influenza H_1N_1 NAs. Interestingly, the new compound **4** showed the strongest inhibitory activity against the neuraminidases from novel influenza H_1N_1 (WT) and oseltamivir-resistant novel H_1N_1 (H274Y mutant) expressed in 293T cells. Although the structure-activity relationships of these compounds were not investigated thoroughly, these results suggest that the C-methylated compounds from *C.opercutus* buds may be a new class of influenza A neuraminidase inhibitors. Moreover, one of the most active isolates obtained in this study, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**5**), was found to be the major component of the EtOAc-soluble extract of *C.opecurlatus* buds. Therefore, this compound may be used as a marker component for quality control of this anti-viral botanical supplement.

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Korea, April, 2010

Nguyen Thi Ngoc Anh

저작물 이용 허락서

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논문제목	한글: 베트남 식물 *Cleistocalyx operculatus* 로부터 분리한 메틸 연결된 플라보노이드 및 그들의 신종플루 뉴라미니데이즈에 대한 저해활성 영문: C-methylated Flavonoids from *Cleistocalyx operculatus* and Their Inhibitory Effects on Novel Influenza A (H1N1) Neuraminidase				

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 내용변경은 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.

2010 년 8월 27일

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