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Xanthones from *Polygala karensium* inhibit neuraminidases from influenza A viruses

Chosun University Graduate School

Department of Pharmacy

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베트남 식물 *Polygala karensium* 부터 인플루엔자 바이러스를 저해하는 잔톤 화합물

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List of Abbreviations

[α]TD: specific rotation

CD: circular dichroism

DMSO: dimethyl sulfoxyde

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

HPLC: high performance liquid chromatography

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

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

IR: infrared absorption

m/z: mass to charge ratio

NA: neuraminidase

NMR: nuclear magnetic resonance

Ppm: parts per million

RP: reverse phase

UV: ultraviolet absorption

WT: wild type

(국문초록)

베트남 식물 *Polygala karensium* 부터 인플루엔자 바이러스를 저해하는 잔톤화합물

당 타이 쭝

지도교수 : 오원근

약학과

조선대학교대학원

세계적 유행병인 H1N1 돼지독감 바이러스의 출현은 신종인플루엔자 A 바이러스의 증가에 의하여 재해 또는 약물내성 바이러스의 발생을 야기할 수 있는 가능성을 가지고 있다. 현재 인플루엔자 뉴라미니다제는 바이러스성 복제, 확산 그리고 발병에 관한 중요 효소로 인플루엔자 퇴치를 위한 가장 유망한 표적 중의 하나로 간주되고 있다. 천연물을 이용한 항 바이러스 탐색 프로그램을 통하여 *Polygala karensium* 의 EtoAc 추출물에서 10 개의 Xantone 유도체를 분리하였다. 분리된 화합물 1, 3, 5, 7 과 9 는 C-1 의 hydroxy 그룹에서 다양한 인플루엔자 바이러스의 변종인 H1N1, H9N2, 신종 H1N1, 그리고 293T cell 에서 발현된 oseltamivir 저항성을 가진 신종 H1N1(H274Y)의 뉴라미니다제에 강한 억제효과를 보였다. 게다가 이러한 화합물들이 MDCK cell 에서 H1N1 돼지독감 바이러스의 세포변성효과를 유도하는 것을 알 수 있었다. 이러한 결과는 *P. Karensium* 로부터 추출한 Xanthone 화합물이 인플루엔자 바이러스의 예방과 치료에 유용하게 사용 될 수 있을 것이라고 제안한다.

1. Introduction

1.1. Influenza and influenza A virus

Influenza, commonly referred to as the flu, is an infectious disease caused by RNA viruses of the family Orthomyxoviridae (the influenza viruses), that affects birds and mammals. The most common symptoms of the disease are chills, fever, sore throat, muscle pains, severe headache, coughing, weakness/fatigue and general discomfort.[1] Although it is often confused with other influenza-like illnesses, especially the common cold, influenza is a more severe disease than the common cold and is caused by a different type of virus.[2]

The influenza viruses of Orthomyxoviridae family can be classified in three types A, B and C.[3] The type A viruses are the most virulent human pathogens among the three influenza types and cause the most severe disease.[4] Influenza A virus can be subdivided into different serotypes based on the antibody response to these viruses.[4] Progenies of the H1N1 influenza A virus that caused the catastrophic and historic pandemic of 1918 have persisted in humans for nearly a century and have continually donated their genes to new viruses, causing new pandemics, epidemics, and epizootics.[5] The 2009 pandemic caused by a novel influenza A (H1N1) virus was derived from two unrelated swine viruses, one of them a derivative of the 1918 human virus, adding to the complexity surrounding this persistent progenitor virus, its descendants, and its several lineages. On June 11, 2009, the World Health Organization (WHO) raised the worldwide pandemic alert to Phase 6 in response to the ongoing global spread of novel influenza H1N1.[5] Although vaccines play a critical role in the prevention of influenza, the efficacy of this intervention can be significantly reduced by a mismatch between the seasonal influenza vaccine and the circulating influenza A virus.[6] Thus, there remains a need for a better control of this infectious agent and antiviral drugs are an important tool in the prevention and management of influenza A virus.

1.2. Position and function of neuraminidase

The influenza A viral particle (Fig. 1) contains a lipid envelope, which is derived from the host's cell membrane during the viral budding process. Three viral proteins, HA, NA, and M2, are embedded in the lipid envelope.[7] HA and NA are spike glycoproteins and they are anchored in the lipid bilayer by the short sequences of hydrophobic amino acids. Electron micrographs of influenza virus show that the HA spike and the NA spike are rod-shaped and mushroom-shaped, respectively. There are 15 known haemagglutinin (H) serotypes and 9 known neuraminidase (N) serotypes. The HA is a homotrimer, which is responsible for the receptor binding and membrane fusion. The NA is a homotetramer whose function is to destroy receptors by hydrolyzing sialic acid groups from glycoproteins and to release the viral progeny.[7] M2 protein is an integral membrane homotetramer, which functions as an ion channel for the acidification of the interior of the viral particle during viral infection.[8,9] Under the viral lipid envelope there is a M1 protein layer.[10]

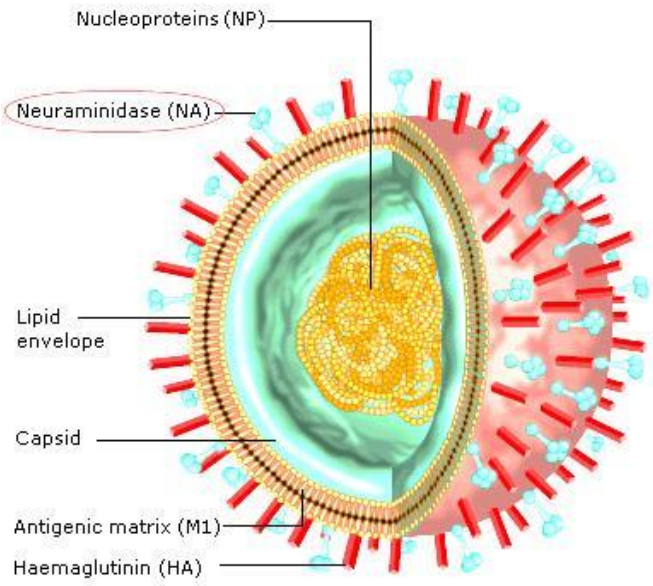


FIGURE 1. Structural diagram of the Influenza virus

1.3. Neuraminidase inhibitors (NAIs)

There are two classes of commercially available agents for influenza treatment: adamantanes and neuraminidase inhibitors. However, adamantanes (amantadine and rimantadine) are not used alone for the treatment of influenza due to their high level of resistance.[11] Neuraminidase inhibitors (NAIs) are effective against all human, avian, and animal influenza viruses.[6,7] They inhibit the release of virions by inhibiting viral NA, which is a key glycoprotein on the surface of the virus.

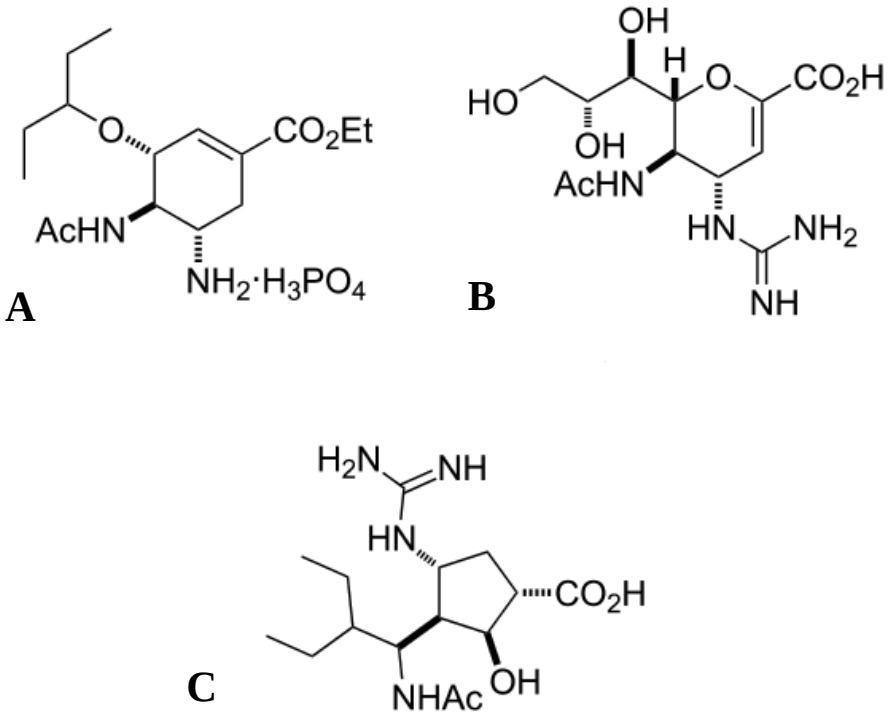


FIGURE 2. Chemical structures of neuraminidase inhibitors.

A –Oseltamivir; B –Zanamivir; C –Peramivir

Three NAIs drugs, oseltamivir, peramivir, and zanamivir (Fig. 2), have been approved worldwide.[12] However, there are reports that adults receiving peramivir and oseltamivir have experience of nausea or vomiting.[13] Furthermore, a high level of drug resistance is caused by the substitutions of a single amino acid in the NA proteins.[9] For these reasons, identification of new antiviral compounds from natural products is important for the development of therapeutic agents against newly appeared influenza virus.[14]

1.4. *Polygala karensium* (Polygalaceae)

Polygala karensium (Fig. 3) belongs to the genus *Polygala* (Polygalaceae) that includes more than 500 species, some of which have traditionally been used to treat amnesia, cough, bronchitis, neurasthenia, and inflammation.[15,16] Triterpenoid derivatives including saponins and xanthones, which have a wide range of biological activities, are reported to be constituents of this genus.[17,18,19]

During the course of an anti-influenza screening program on natural products, the EtOAc-soluble extract of *P. karensium* was found to have NA inhibitory activities. This prompts to phytochemically examine the molecular constituents responsible for the NA inhibitory activities using *in vitro* assays as bioactivity-guided fractionation. This thesis describes the isolation, structural elucidation and antiviral activities of these compounds on NAs from two influenza viral strains, H1N1 and H9N2, as well as from both novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells.

FIGURE 3. *Polygala karensium*– 1. Flowering branch; 2. Flower; 3. Petals spread out, showing stamens; 4. Inner sepal; 5. Upper outer sepal; 6. Lateral outer sepal; 7. Pistil; 8. Fruit; 9. Seed.

2. Materials and Methods

2.1. Materials

2.1.1. Plant material

The dried roots of *Polygala karensium* were collected from a medicinal garden in September 2010 in Lao Cai province, Vietnam, and were identified botanically by Dr. Nguyen Thi Bich Thu. A voucher specimen (NIMM2010–21) has been deposited at the Herbarium of the National Institute of Medicinal Materials, Hanoi, Vietnam.

2.1.2. Chemicals, reagents and chromatography

Silicagel (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 Gibson system with a UV detector and an Optima Pak C18 column (10 × 250 mm, 10 μm particle size, RS Tech, Korea). NMR spectra wered obtained on a Varian Inova 300 MHz spectrometer with TMS as the internal standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). All solvents used for extraction and isolation were analytical grade. The EIMS data was performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer.

Nicotinamide, tamoxifen were purchased from Sigma Chemical Company (St Louis, MO, USA). Oseltamivir phosphate was purchased from Hoffman-La Roche Ltd, Basel, Switzerland. Dulbecco's modified Eagle′s medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from GIBCO-BRL (Grand Island, NY, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)] was purchased from USB Corporation (Cleveland, OH). Welfect EX-plus was purchased from Welgen, Inc (Deagu, Korea).

2.2. Methods

2.2.1. Extraction and isolation

Bioassay-guided isolation of compounds **1**–**10**: 2 kg of the dried roots of *Polygala karensium* were extracted with MeOH (3 × 4.0 L) at room temperature for a week. The combined methanol extracts were then concentrated to yield a dry residue (230.0 g). This crude extract was suspended in H_2O (2.0 L) and partitioned successively with *n*-hexane (3 × 1.5 L), EtOAc (3 × 1.5 L), and *n*-BuOH (3 × 1.5 L). The EtOAc fraction (46.0 g), which showed strong H1N1 influenza NA inhibitory activity (IC_{50} = 38.24 ± 2.78 μg/mL), was chromatographed over a silica gel column (10 × 25 cm; 63–200 μm particle size) and eluted with gradient solvent *n*-hexane/EtOAc (9:1, 8:2…1:9, each 2.5 L) to yield six fractions (F1: 7.2 g; F2: 5.2 g; F3: 4.5 g; F4: 4.8 g; F5: 6.7 g; F6: 9.6 g) based on TLC profile. The active fractions, F2, F4, and F5, were subjected to additional chromatography. Fraction F2 was applied to an RP-18 column (5 × 30 cm; 40–63 μm particle size) with a stepwise gradient of MeOH/H_2O (3:1 to 10:1) to afford six subfractions (F2.1–F2.6). Fraction F2.3 (120 mg) was further separated by HPLC [Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeOH in H_2O containing 0.1% HCO_2H (0–65 min: 64% MeOH, 65–70 min: 64–100% MeOH, 70–80 min: 100% MeOH); flow rate 2 mL/min; UV detection at 205 and 254 nm] to give compound **1** (t_R = 45.5 min, 5.5 mg) and compound **2** (t_R = 58.0 min, 8.0 mg). Fraction F2.4 (560 mg) was purified by a Sephadex LH-20 column (4 × 25 cm) using MeOH as the eluting solvent to yield compound **3** (145.0 mg). Fraction F4 was applied to a RP-18 column (5 × 30 cm; 40–63 μm particle size) and eluted with a stepwise gradient of MeOH/H_2O (1:1 to 10:1) to afford seven subfractions (F4.1–F4.7). Further separation of F4.4 (160.0 mg) by HPLC (0–55 min: 50% MeOH, 60 min: 100% MeOH) resulted in the isolation of compound **4** (t_R= 45.0 min, 7.5 mg) and compound **5** (t_R= 51.0 min, 22.0 mg). Fraction F5 was chromatographed over a Sephadex LH-20 column (7 × 30 cm) using MeOH as the eluting solvent

to afford four subfractions (F5.1–F5.4). From subfraction F5.2 (180.0 mg), compound **6** (t_R= 32.0 min, 10.5 mg) and compound **7** (t_R= 43.0 min, 11.0 mg) were afforded by HPLC (0–45 min: 45% MeOH, 50 min: 100% MeOH). Finally, purification of fraction F5.3 (160.0 mg) on HPLC (0–55 min: 66% MeOH, 60 min: 100% MeOH) yielded compound **8** (t_R= 45.0 min, 5.0 mg), compound **9** (t_R= 50.0 min, 8.5 mg), and compound **10** (t_R= 52.0 min, 6.5 mg).

2.2.2. Cloning of novel H1N1 influenza neuraminidase

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) (Gibco) supplemented with 10% fetal bovine serum at 37°C and 5% CO2. 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 10^4 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 μgWelfect-Ex plus reagent for 6 hrs at 37°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. Neuraminidase 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, 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 inhibiting 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, 0.1M 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 reduction assay

H1N1 influenza virus was inoculated onto near confluent MDCK cell monolayers (1×10^4 cells/well) by replacing medium culture with 100 μL virus solution diluted in infection medium, DMEM containing 10 μg/mL trypsin, at the concentration of 50 $TCID_{50}$/100μL. After one hour, 100 μL of infection medium and isolated compounds at different concentrations were added into each well. The cultures were incubated for 4 days at 37°C under 5% CO_2 atmosphere. CPE effect was assessed through the cell viabilities and was determined by the method of MTT assay

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

2.2.7. MTT assay for the estimation of cell viability and cytotoxicity of compounds

The MDCK cells were grown in 96-well plates with 10^4 cells per well for 24 hrs. The plates were replaced with media containing the serially diluted compounds. After 48 hours incubation, a solution of 40 µL of 2 mg/mL MTT (Sigma-Aldrich Co. M-2128) in 1X PBS was added to each well. The cells then were incubated further for 3-6 hours at 37°C, the medium then was changed into 200 µL of DMSO and the optical density (OD) of the wells was determined at a test wavelength of 550 nm using VersaMax ELISA microplate reader. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis.

2.2.8. Statistical analysis

A statistical calculation was carried out using Microsoft Excel 2010 and Sigma Plot 11.0. The results are expressed as the mean ± SD of three to five independent experiments.

3. Results and discussions

3.1. Isolation of compounds from roots of *P. karensium*

In order to isolate the compounds with inhibitory activity against influenza neuraminidase, the EtOAc-soluble extract of *P. karensium* was subjected to a succession of chromatographic procedures, including silica gel chromatography, Shephadex LH-20, RP-C18, and HPLC to afford ten xanthone derivatives (**1–10**) as the active principles (Fig. 4).[6] The result of analysis by HPLC of the EtOAc-soluble extract was shown in Fig. 2 that retention times (t_R, min) of compounds **1–10** were 32.0, 39.0, 40.5, 27.5, 31.2, 13.0, 19.5, 34.5, 37.5, and 38.2 min, respectively.

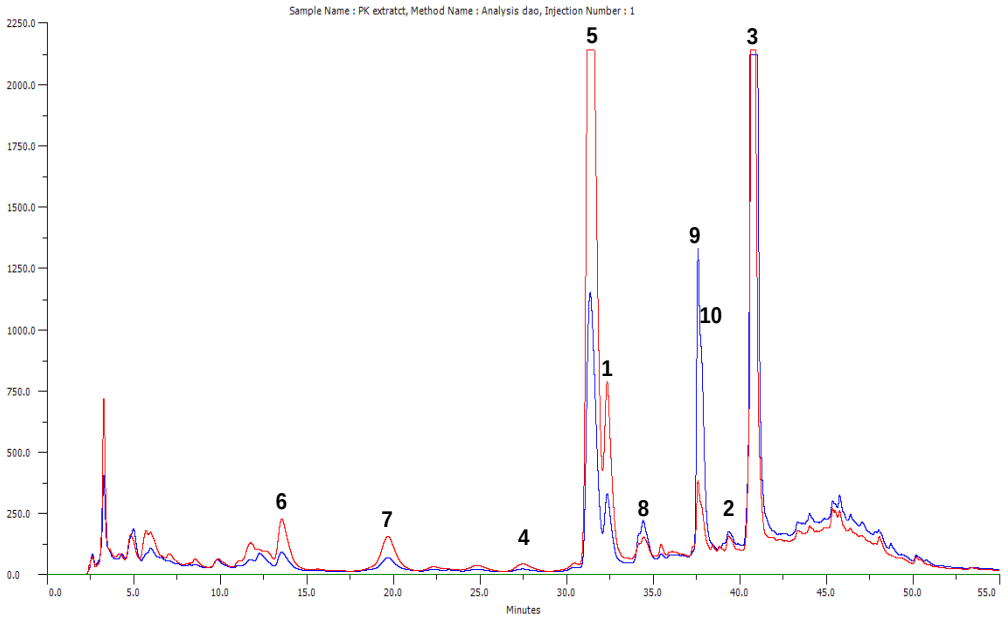
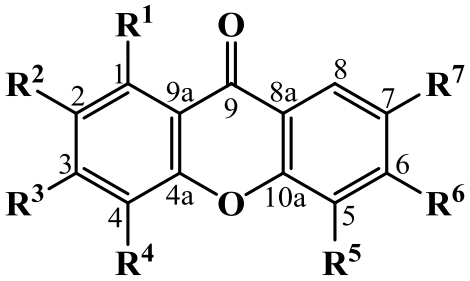
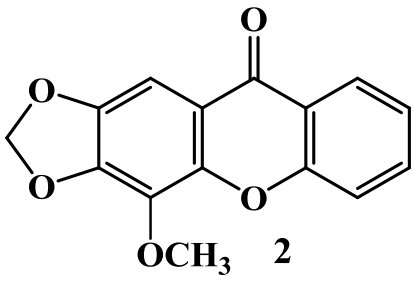


FIGURE 4. A representative HPLC profile of compounds **1 – 10** from the EtOAc-soluble extract of the roots of *P. karensium*. HPLC was performed with an YMC–C18 column (250 × 4.6 mm ID, particle size 6 μm, Japan). The detection wavelengths were 205 (blue line) and 254 (red line) nm. Elution was carried out with MeOH in H2O (0–40 min: 50 % MeOH; 40–45 min: 50–100 % MeOH; 45–55 min: 100 % MeOH) at flow rate of 1 mL/min.

3.2. Structure determination of isolated compounds

Compound **1**, obtained as yellow needles, mp 259–260°C, had a molecular formula of $C_{13}H_8O_4$ as established by the EIMS ($[M]^+$ at *m/z* 228) and ^{13}C NMR spectra. The UV absorption bands (238 and 325 nm) and the ^1H NMR signals [δ_H:6.27 (1H, s, H-2), 6.44 (1H, s, H-4), 7.53 (1H, d, J = 8.5 Hz, H-5), 7.84 (1H, t, J = 8.0 Hz, H-6), 7.46 (1H, t, J = 8.0 Hz, H-7), and 8.20 (1H, d, J = 8.5 Hz, H-8)] suggested a xanthone skeleton for **1**.[21,22,23] These assignments were identical with those reported for 1,3-dihydroxyxanthone.[17] In the same way, the UV, EIMS, and NMR data of compounds **2–10** were in good agreement with those previously reported for 4-methoxy-2,3-methylenedioxyxanthone (**2**),[24] 1,7-dihydroxyxanthone (**3**),[7a,8] 3,4-dimethoxy-2-hydroxyxanthone (**4**),[25] 1,7-dihydroxy-4-methoxyxanthone (**5**),[26] 3,6-dihydroxy-1,2-dimethoxyxanthone (**6**),[27] 1,3,7-trihydroxyxanthone (**7**),[28] 1,2-dimethoxy-4-hydroxyxanthone (**8**),[29] 1,2,3,5-tetrahydroxyxanthone (**9**)[30] and 7-hydroxy-1-methoxyxanthone (**10**)[18] respectively. To our knowledge, this is the first report of the isolation of these xanthones (**1–10**) from *P. karensium*.





	R^1	R^2	R^3	R^4	R^5	R^6	R^7
1	OH	H	OH	H	H	H	H
3	OH	H	H	H	H	H	OH
4	H	OH	OCH_3	OCH_3	H	H	H
5	OH	H	H	OCH_3	H	H	OH
6	OCH_3	OCH_3	OH	H	H	OH	H
7	OH	H	OH	H	H	H	OH
8	OCH_3	OCH_3	H	OH	H	H	H
9	OH	OH	OH	H	OH	H	H
10	OCH_3	H	H	H	H	H	OH

FIGURE 5. Chemical structure of compounds **1 – 10** isolated from *Polygala karensium*

1,3-Dihydroxyxanthone (1): yellow needles; mp 259–260°C; UV(MeOH) λ_{max}238, 325 nm; EIMS *m/z* 228 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 12.90 (s, OH-1), 8.20 (1H, d, *J* = 8.4 Hz, H-8), 7.84 (1H, t, *J* = 8.4 Hz, H-6), 7.53 (1H, d, *J* = 8.4 Hz, H-5), 7.46 (1H, t, *J* = 8.4 Hz, H-7), 6.44 (1H, s, H-4), 6.27 (1H, s, H-2).

4-methoxy-2,3-methylenedioxyxanthone (2): yellow needles; mp 232–233°C; EIMS *m/z* 270 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.15 (1H, d, *J* = 8.4 Hz, H-8), 7.75 (1H, t, *J* = 8.4 Hz, H-6), 7.47 (1H, d, *J* = 8.4Hz, H-5), 7.39 (1H, t, *J* = 8.4 Hz, H-7), 6.82 (1H, s, H-1), 6.18 (2H, s, O_2CH_2-2,3), 4.02 (3H, s, OCH_3-4).

1,7-Dihydroxyxanthone (3): yellow needles; mp 195–196°C; UV(MeOH) λ_{max}232, 309 nm; EIMS *m/z* 228 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 12.71 (s, OH-1), 7.68 (1H, t, *J* = 8.4 Hz, H-3), 7.58 (1H, d, *J* = 2.4 Hz, H-8), 7.50 (1H, d, *J* = 8.4Hz, H-5), 7.40 (1H, dd, *J* = 8.4, 2.5 Hz, H-6), 6.97 (1H, d, *J* = 8.4 Hz, H-4), 6.74 (1H, d, *J* = 8.4 Hz, H-2).

3,4-dimethoxy-2-hydroxyxanthone (4): yellow needles; EIMS *m/z* 272 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.18 (1H, d, *J* = 8.4 Hz, H-8), 7.75 (1H, t, *J* = 8.4 Hz, H-6), 7.46 (1H, d, *J* = 8.4 Hz, H-5), 7.40 (1H, t, *J* = 8.4 Hz, H-7), 6.78 (1H, s, H-1), 3.95 (3H, s, OCH_3-4), 3.87 (3H, s, OCH_3-3).

1,7-Dihydroxy-4-methoxyxanthone (5): yellow needles; mp 239–240°C; UV(MeOH) λ_{max}236, 324 nm; EIMS *m/z* 258 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 12.11 (s, OH-1), 7.58 (1H, s, H-8), 7.56 (1H, d, *J* = 8.4 Hz, H-5), 7.42 (2H, d, *J* = 8.4Hz, H-3, H-6), 6.68 (1H, d, *J* = 8.4 Hz, H-2), 3.95 (3H, s, OCH_3-4).

3,6-dihydroxy-1,2-dimethoxyxanthone (6): brown needles; EIMS *m/z* 288 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.04 (1H, d, *J* = 8.4 Hz, H-8), 7.52 (1H, d, *J* = 8.4 Hz, H-7), 7.29 (1H, s, H-5), 6.21 (1H, s, H-4), 3.90 (3H, s, OCH_3-2), 3.88 (3H, s, OCH_3-1).

1,3,7-Trihydroxyxanthone (7): yellow needles; mp 298–299°C; UV(MeOH) λ_{max}233, 308 nm; EIMS *m/z* 244 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 12.98 (s, OH-1), 7.56 (1H, d, *J* = 2.4 Hz, H-8), 7.42 (1H, d, *J* = 8.4 Hz, H-5), 7.36 (1H, dd, *J* = 8.4, 2.4 Hz, H-6), 6.49 (1H, s, H-4), 6.25 (1H, s, H-2).

1,2-dimethoxy-4-hydroxyxanthone (8): yellow needles; mp 200–202°C; UV(MeOH) λ_{max}237, 350 nm; EIMS *m/z* 272 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 8.17 (1H, d, *J* = 8.4 Hz, H-8), 7.73 (1H, t, *J* = 8.4 Hz, H-6), 7.55 (1H, d, *J* = 8.4 Hz, H-5), 7.40 (1H, t, *J* = 8.4 Hz, H-7), 6.51 (1H, s, H-3), 3.93 (3H, s, OCH_3-2), 3.88 (3H, s, OCH_3-1).

1,2,3,5-Tetrahydroxyxanthone (9): yellow needles; mp 264–265°C; UV(MeOH) λ_{max} 250, 325 nm; EIMS *m/z* 260 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 12.98 (s, OH-1), 7.56 (1H, m, H-7), 7.43 (1H, m, H-8), 7.40 (1H, m, H-6), 6.26 (1H, s, H-4).

7-hydroxy-1-methoxyxanthone (10): yellow needles; mp 239–240°C; UV(MeOH) λ_{max}242, 373 nm; EIMS *m/z* 242 $[M]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 7.68 (1H, t, *J* = 8.4 Hz, H-3), 7.58 (1H, d, *J* = 2.4 Hz, H-8), 7.41 (1H, d, *J* = 8.4 Hz, H-5), 7.28 (1H, dd, *J* = 8.4, 2.4 Hz, H-6), 7.06 (1H, d, *J* = 8.4 Hz, H-4), 6.93 (1H, d, *J* = 8.4 Hz, H-2), 3.95 (3H, s, OCH_3-1).

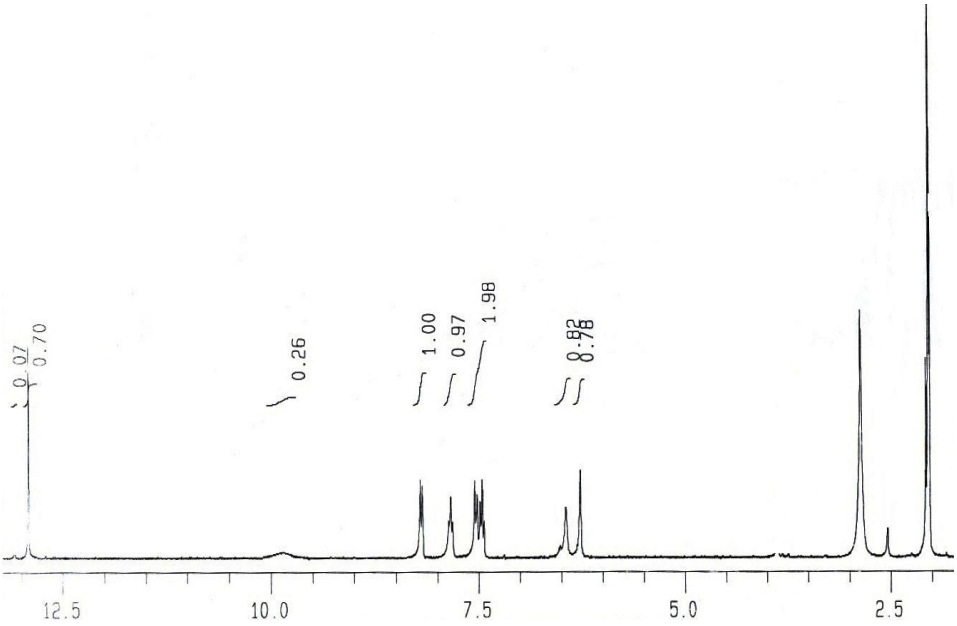


FIGURE 6. ^{1}H NMR (300 MHz, acetone-d_6) of compound **1**

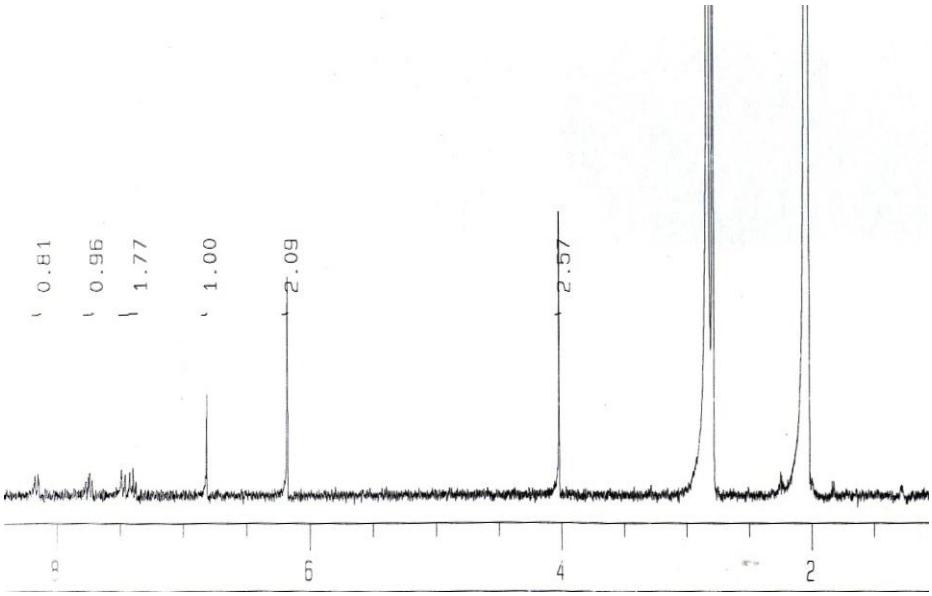


FIGURE 7. ^{1}H NMR (300 MHz, acetone-d_6) of compound **2**

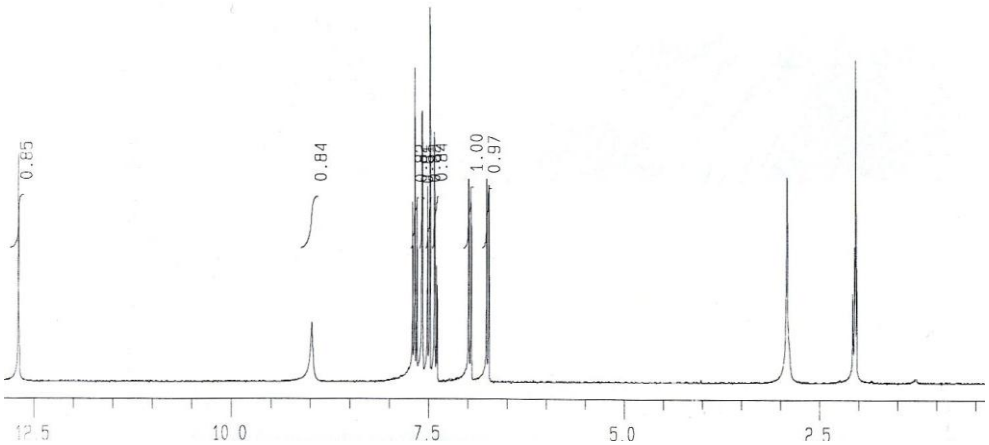


FIGURE 8. ^{1}H NMR (300 MHz, acetone-d_6) of compound **3**

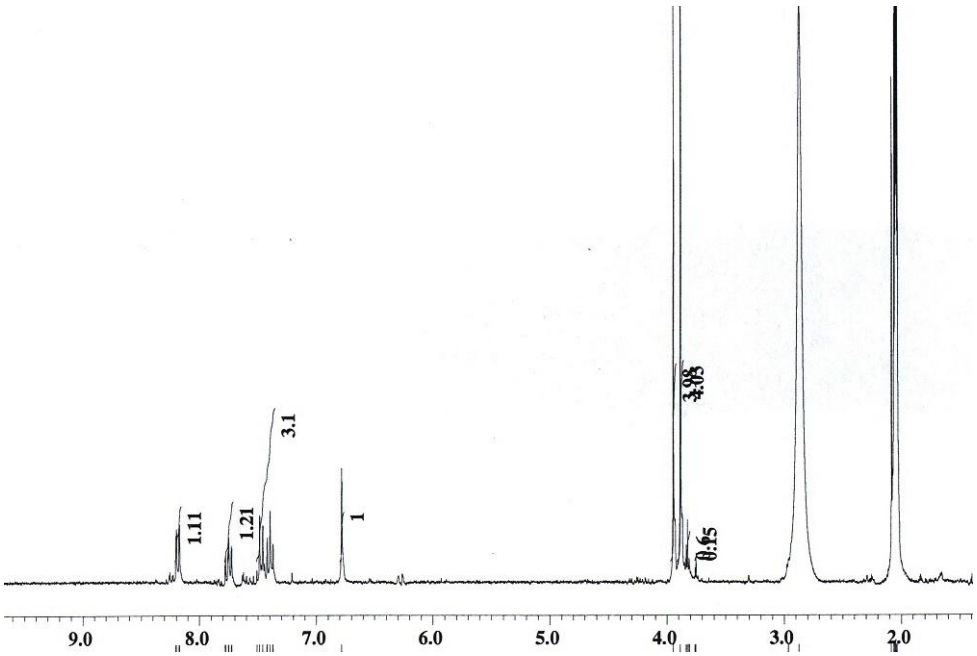


FIGURE 9. ^{1}H NMR (300 MHz, acetone-d_6) of compound **4**

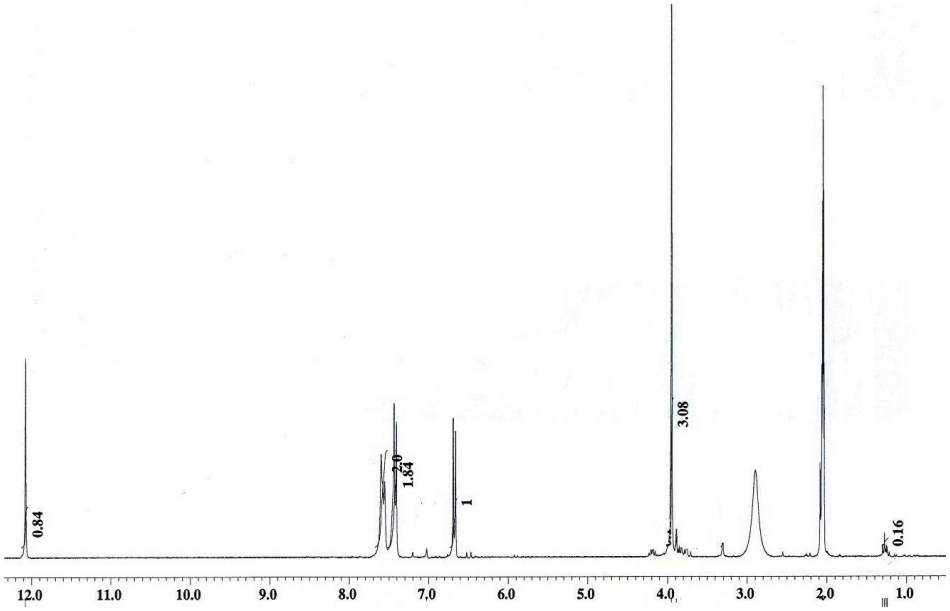


FIGURE 10. ^{1}H NMR (300 MHz, acetone-d_6) of compound **5**

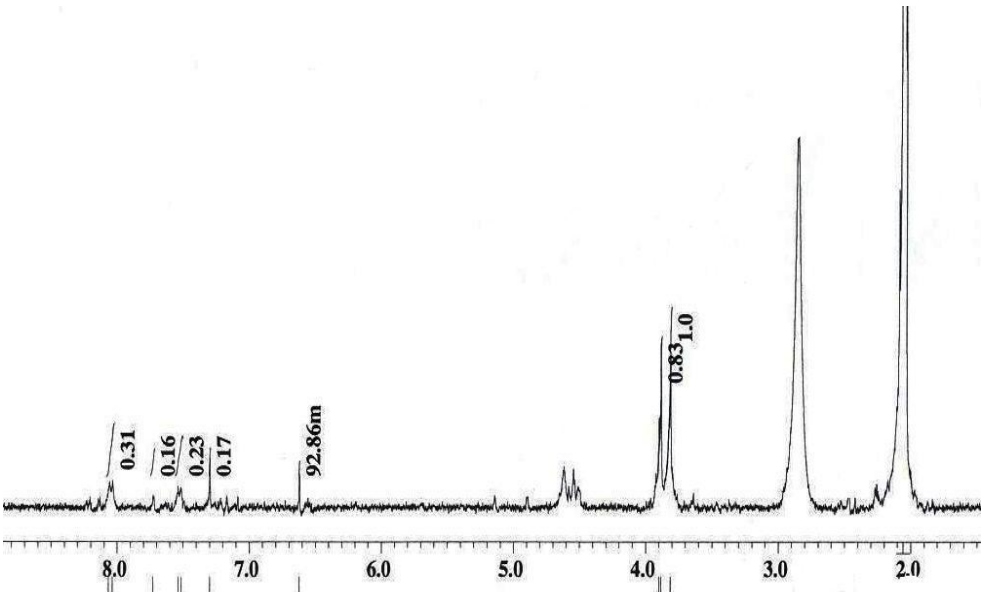


FIGURE 11. ^{1}H NMR (300 MHz, acetone-d_6) of compound **6**

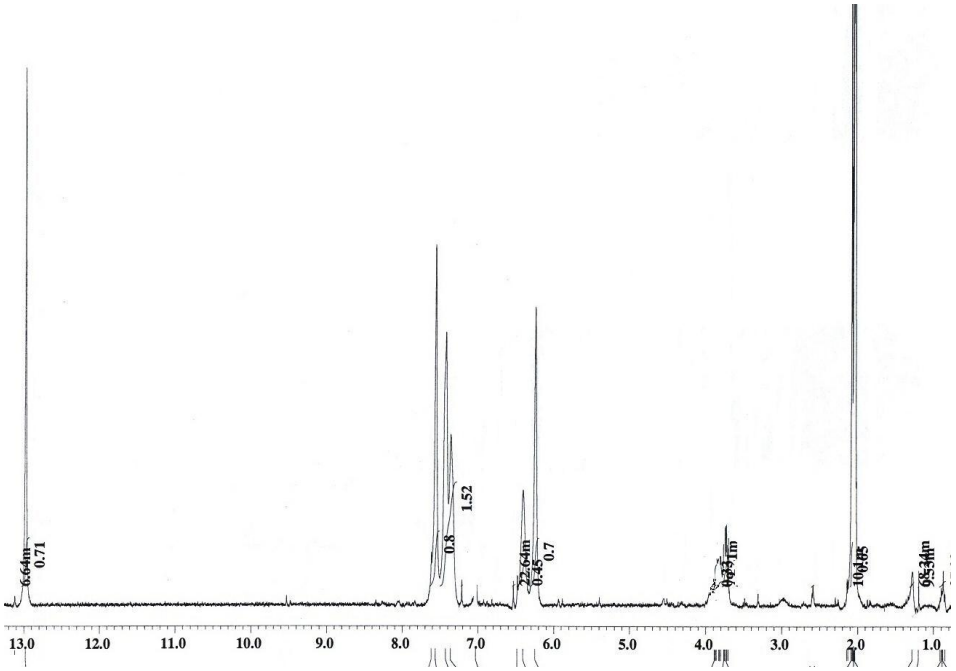


FIGURE 12. ^{1}H NMR (300 MHz, acetone-d_6) of compound **7**

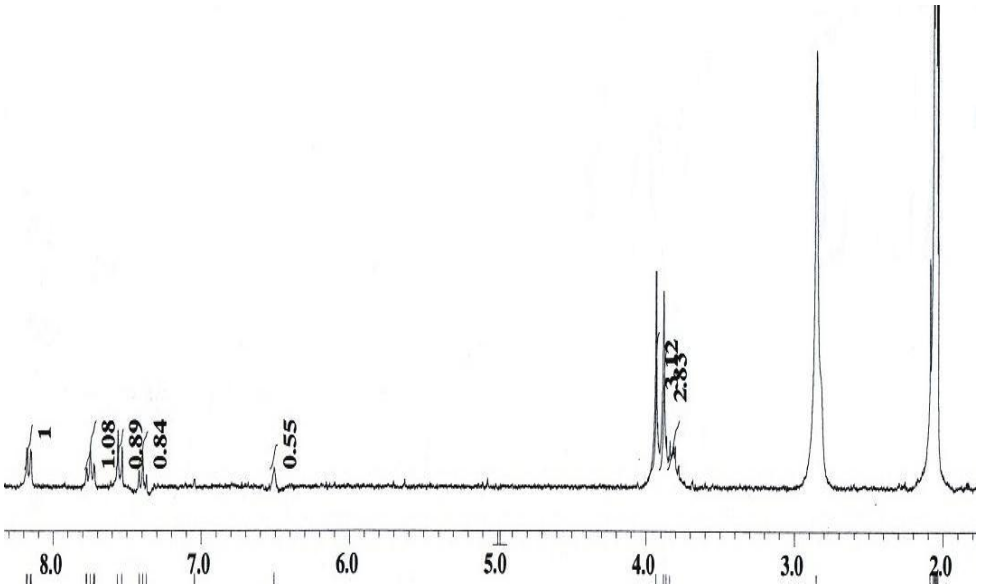


FIGURE 13. ^{1}H NMR (300 MHz, acetone-d_6) of compound **8**

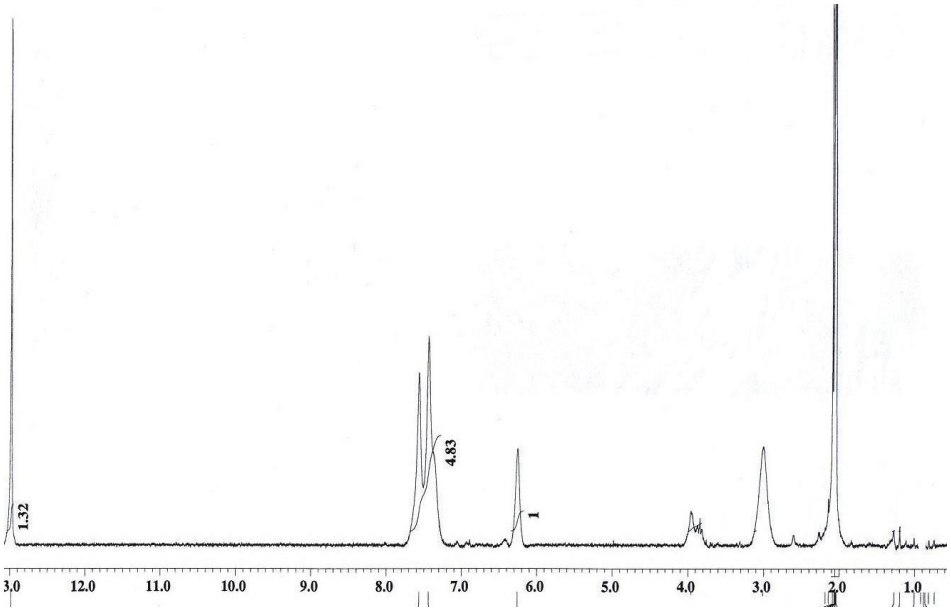


FIGURE 14. ^{1}H NMR (300 MHz, acetone-d_6) of compound **9**

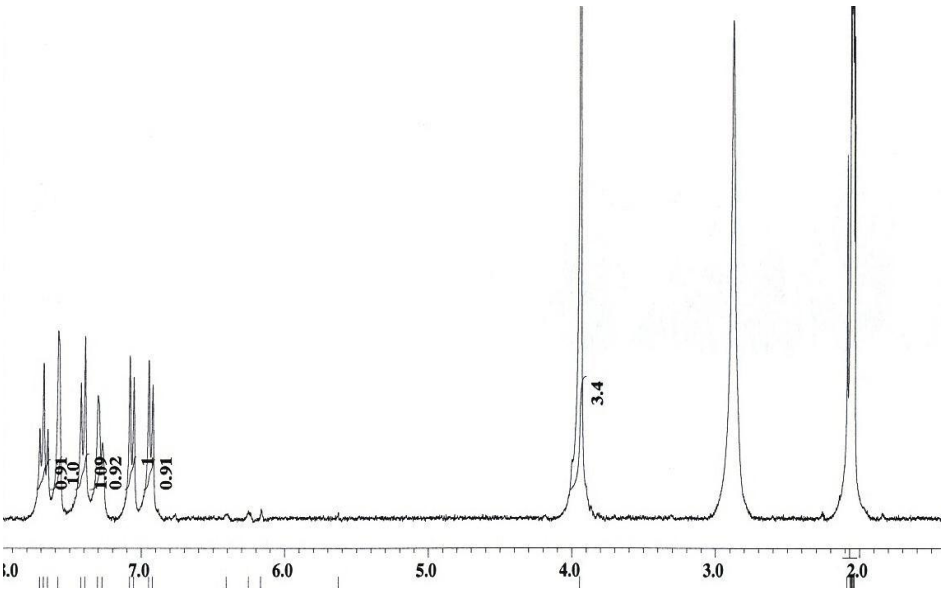


FIGURE15. ^{1}H NMR (300 MHz, acetone-d_6) of compound **10**

TABLE 1.^{13}C NMR Spectroscopic Data[a] for compounds 1, 3, 5, 7 and 9

Position	**1**	**3**	**5**	**7**	**9**
1	164.8	162.7	155.4	164.7	150.2
2	99.1	110.5	108.8	98.8	136.3
3	166.6	137.8	121.2	166.4	152.5
4	94.9	107.8	141.2	94.6	99.5
4a	158.9	157.3	149.0	159.1	152.0
5	118.6	120.2	120.5	120.0	149.2
6	136.4	126.1	126.3	125.1	119.1
7	126.4	154.9	155.2	154.9	122.5
8	125.2	121.8	121.9	121.9	118.3
8a	120.5	109.1	109.1	109.4	120.1
9	181.4	182.9	182.9	181.3	181.2
9a	103.9	109.1	109.7	103.6	102.9
10a	156.9	151.0	151.0	150.8	148.4
4-OCH_3			57.6		

[a]Compounds were measured at 75 MHz in CD_3COCD_3.

3.3. Effects of the isolated compounds on NAs from two influenza viral strain, H1N1 and H9N2

All isolated compounds were examined for their inhibitory activity against NAs from influenza viruses. Oseltamivir phosphate (Hoffman-La Roche Ltd, Basel, Switzerland) was used as a positive control.[31] The assay was performed as described by measuring the hydrolysis of 4-methylumbelliferyl-α-D-N-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) via fluorescence. As shown in Table 2 and Fig. 16A-B, these compounds showed inhibitory activity against NAs from H1N1 and H9N2 influenza viruses in a dose-dependent manner. Compounds **1**, **3**, **5**, **7**, and **9** with a hydroxyl group at carbon C-1 exhibited high inhibitory activity (with IC_{50} values ranging from 23.29 ± 2.08 to 28.42 ± 1.47 and 15.46 ± 1.69 to 25.59 ± 2.14 μg/mL, respectively), whereas the other compounds without this hydroxyl group had lower activity (IC_{50}> 100 μg/mL).

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

This study next examined whether the isolates effectively inhibited NAs from the wild-type novel swine flu (WT) virus and the oseltamivir-resistant virus.[32] As shown in Table 1 and Fig. 17A-B, the recombinant NA mutant protein H274Y, which was resistant to oseltamivir (243-fold decrease in IC_{50} value compared to novel H1N1), was susceptible to all compounds. Interestingly, tested compounds showed stronger activity against the H274Y mutant form and the novel H1N1 form than the original H1N1 form (from two to three fold decreases in IC_{50} values).

TABLE 2. Inhibitory effects of compounds 1-10 on the neuraminidase activity

Compound	IC_{50} (μg/mL)[a]			
	H1N1	**H9N2**	**H1N1(WT)**	**H1N1 (H274Y)**
1	23.29 ± 2.08	15.46 ±1.69	11.15 ± 0.52	7.73 ± 0.85
2	> 100	> 100	NT[b]	NT[b]
3	23.54 ± 3.68	22.45 ± 3.45	11.54 ± 0.35	13.01 ± 0.41
4	> 100	> 100	NT[b]	NT[b]
5	28.42 ± 1.47	25.59 ± 2.14	9.33 ± 0.6	12.8 ± 1.07
6	> 100	> 100	NT[b]	NT[b]
7	26.81 ± 2.18	24.77 ± 2.45	13.41 ± 1.09	9.14 ± 0.39
8	> 100	> 100	NT[b]	NT[b]
9	26.87 ± 3.81	19.81 ± 3.34	13.68 ± 0.89	10.80 ± 0.48
10	> 100	> 100	NT[b]	NT[b]
Oseltamivir	39.74 ± 1.54 (ng/mL)	4.94 ± 0.56 (ng/mL)	21.09 ± 1.19 (ng/mL)	5.13 ± 0.23

[a] All compounds were examined in a set of triplicated experiments. [b] NT: not tested.

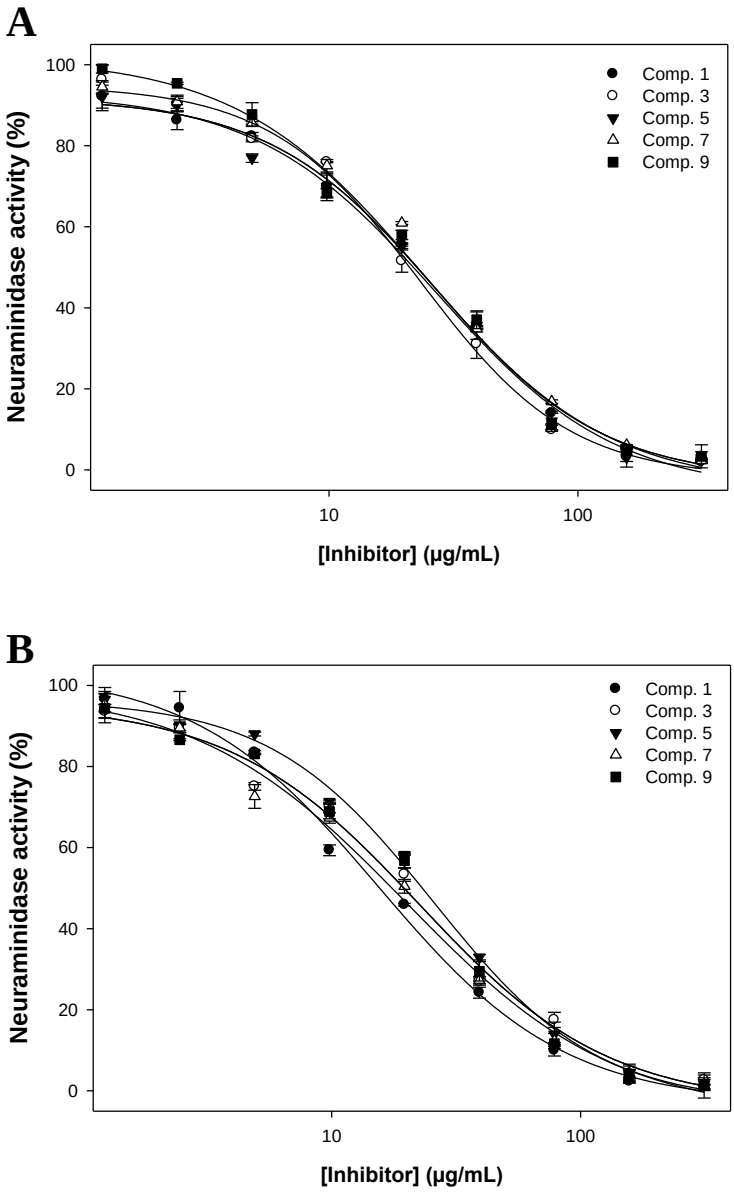


FIGURE 16 (A-B). Effects of compounds **1, 3, 5, 7** and **9** on the activity of NAs from influenza A H1N1 and H9N2 for the hydrolysis of 4-MU-NANA at 37 °C. Inhibitor concentrations are displayed on logarithmic scales. The IC_{50} is identified from the midpoint (neuraminidase activity = 50%) of the semi-log plot.

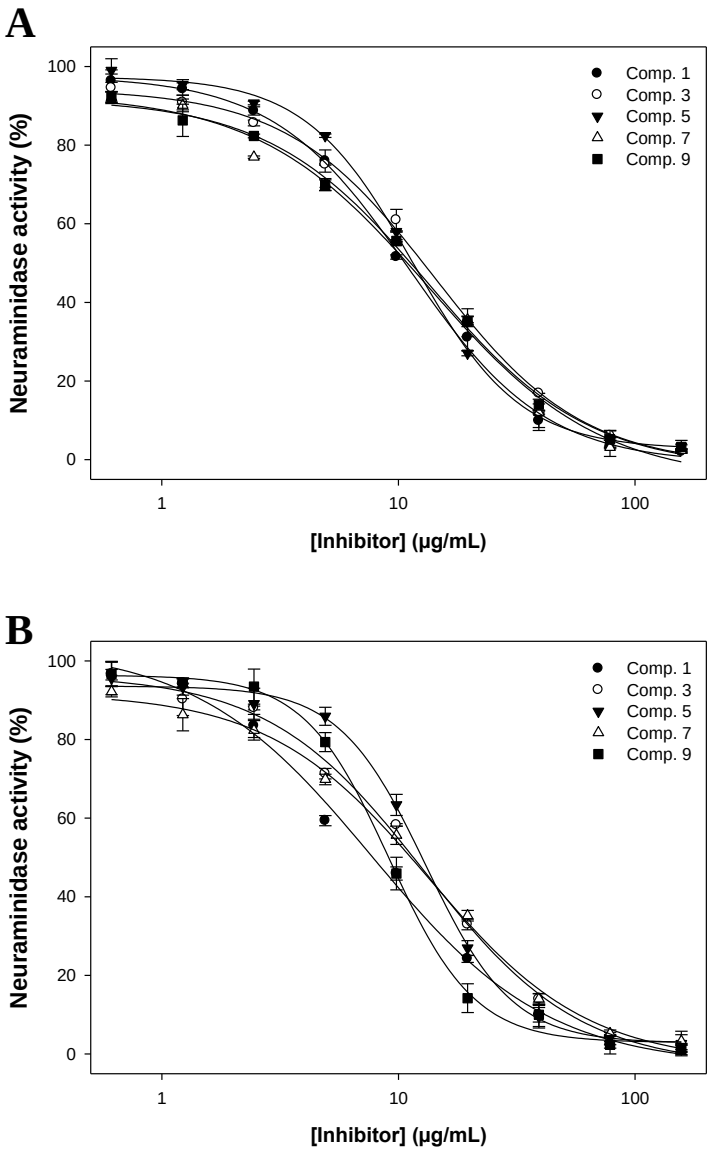


FIGURE 17 (A-B). Effects of compounds **1, 3, 5, 7** and **9** on the activity of NAs from influenza A novel H1N1 and oseltamivir-resistant novel H1N1 (H274Y) for the hydrolysis of 4-MU-NANA at 37 °C. Inhibitor concentrations are displayed on logarithmic scales. The IC_{50} is identified from the midpoint (neuraminidase activity = 50%) of the semi-log plot.

3.5. Inhibition pattern of the compounds on neuraminidase of H1N1

To study the mode of inhibition, both the double reciprocal Lineweaver-Burk and Dixon plots were used (Fig.18A-B). As shown in Figure 15A-B, increasing the substrate concentrations resulted in a family of lines that did not intersect on the *y*-axis in the Lineweaver-Burk plot but intersected at a non-zero point on the negative *x*-axis ($-K_i$) in Dixon plots. These results suggest that these compounds are noncompetitive inhibitors.

3.6. 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

The inhibitory effect of compounds **1**, **3**, **5**, **7**, and **9** on replication of H1N1 influenza A PR/8/34 virus was further assessed by the cytopathic effect (CPE) reduction assay. CPE refers to degenerative changes in cells associated with the multiplication of certain viruses, thus the antiviral activity of potential compounds can be determined by evaluating the inhibition of virus-induced cell death.[33,34,35] Whereas cells without inhibitors were completely destroyed by the virus, cell survival was increased by the compounds at a final concentration of 5 μg/mL (Fig. 19A). Treatment with compound **3** after virus infection showed a clearly protective effect against virus infection of xanthone in Fig. 19B and Fig. 20, respectively. Besides, a MTT assay was carried out for the cytotoxic of these compounds and the results showed that all compounds are non-toxic for MDCK cells.

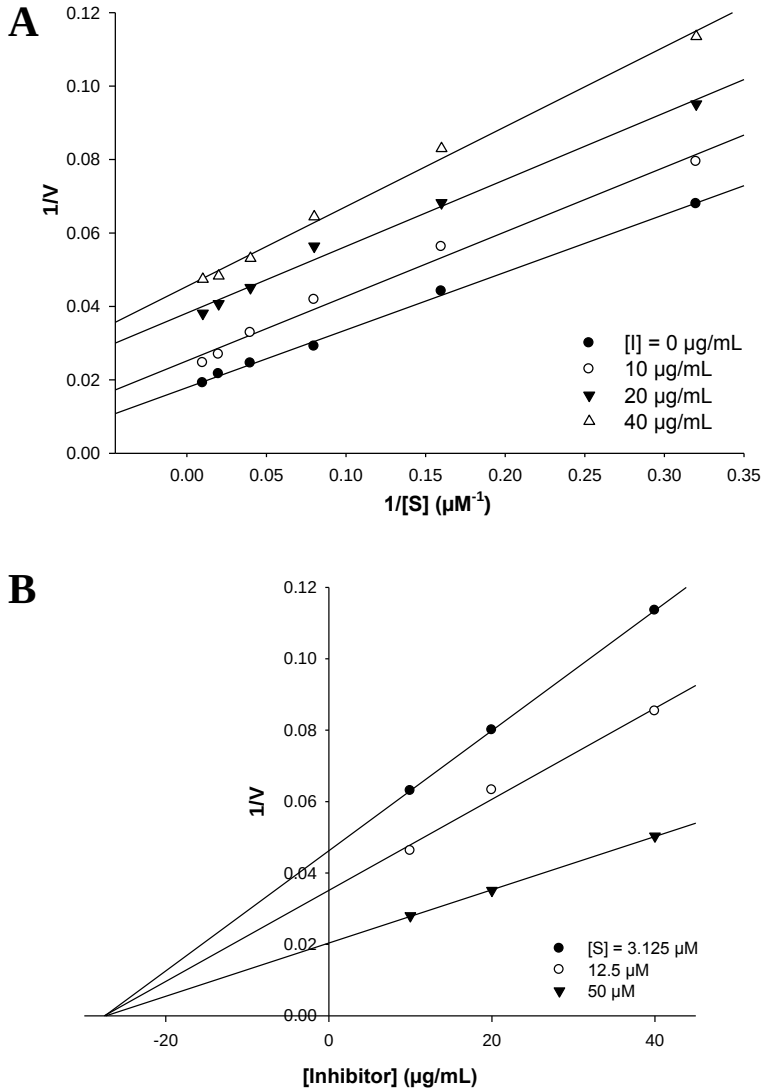


FIGURE 18 (A-B). Graphical determination of the type of inhibition for compound **3**. (A) Lineweaver-Burk plot for the inhibition of compound **3** on NA from influenza A (H1N1). The data is expressed as the mean reciprocal of the intensity/min for n = 3 replicates at each substrate concentration. (B) Dixon plot for compound **3** determining the inhibition constant K_i. The K_i value is determined from the negative of the *x*-axis value at the point of the intersection of the three lines. The data is expressed as the mean reciprocal of the intensity/min of n = 3 replicates at each substrate concentration.

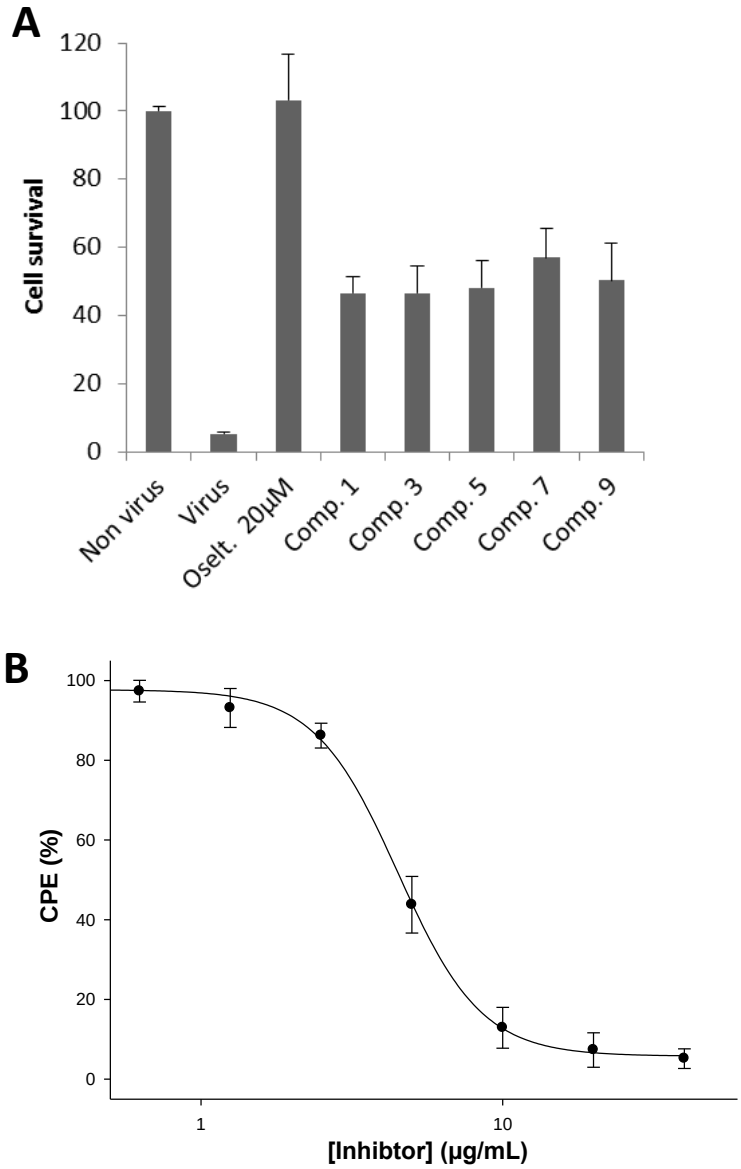


FIGURE 19 (A-B). Inhibitory activities of compounds **1**, **3**, **5**, **7** and **9** against CPE of H1N1 influenza A virus in MDCK cells. (A) Cell survival is increased by the compounds at final concentration of 5 μg/mL. (B) Dose-dependent effect of compound **3** reduces CPE of H1N1 virus in MDCK cells.

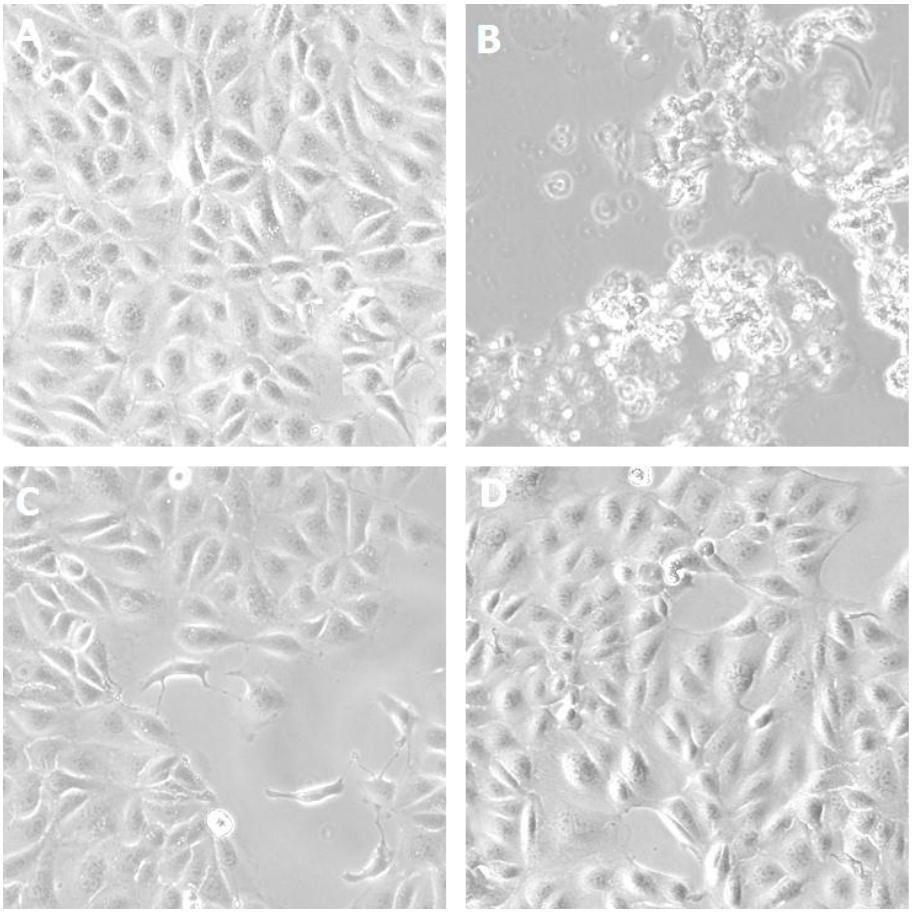


FIGURE 20. Morphologies of MDCK cells show the effects of compound **3** on H1N1-induced CPE. (A) Non-infected cells; (B) H1N1-infected cells without compound **3**; (C) H1N1-infected cells with 5 μg/mL of compound **3** (21.93 μM); (D) H1N1-infected cells with 2 μg/mL of oseltamivir.

4. Conclusions

Some previous studies have reported that xanthone derivatives have NA inhibitory activity in bacteria.[36,37] However, the effects of xanthones on influenza NA and their structure activity relationships have not been studied so far. Moreover, the role of the prenyl group in the inhibition of NAs from bacteria and influenza virus is completely different.[26] Drug-resistant influenza viruses and the threat of a new pandemic highlight the need for novel and effective antiviral agents.[38] The present study showed that five out of 10 xanthone derivatives from *P. karensium* exhibited strong inhibition against the NAs of influenza H1N1 viruses. Although the structure activity relationships of these xanthones have not been investigated thoroughly, our results suggest that the hydroxyl group at C-1 is very important for increasing the activity. Moreover, one of the most active isolates, 1,7-dihydroxyxanthone (**3**), was the major component of the EtOAc-soluble extract of this plant. Therefore, this compound can be used as a marker component for quality control of this antiviral botanical supplement. Additionally, syntheses of these naturally-occurring compounds and their analogs may provide an opportunity for the development of new therapeutic agents for this serious disease.

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논문제목	**한글:** 베트남 식물 Polygala karensium 부터 인플루엔자 바이러스를 저해하는 잔톤 화합물 **영문:**Xanthones from *Polygala karensium*inhibit neuraminidases from influenza A viruses				

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

- 다 음 -

1. 저작물의 DB 구축 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 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.

5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 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.

2012 년 8 월 24 일

동의 여부: 동의 (O) 조건부 동의( ) 반대 ( )

저작자: 당 타이 쭝 (서명 또는 인)

조선대학교 총장 귀하