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Neuraminidase Inhibitors from *Reynoutria elliptica*

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In the course of screening neuraminidase inhibitors from herbal medicines, *Reynoutria elliptica* exhibited high inhibitory activity. Four active compounds were isolated from the ethyl acetate soluble fraction by consecutive purification using sillica gel, Sephadex LH-20 chromatography, and recrystallization. The chemical structures of these compounds were identified as 1,3,8-trihydroxy-6-methylanthraquinone (emodin) 1,8-dihydroxy-3-methoxy-6-methylanthraquinone (emodin 3-methyl ether; physcion), 1,3,8-trihydroxy-6-hydoxymethylanthraquinone (ω-hydroxyemodin), and 3,5,4'-trihydroxystilbene (*trans*-resvertrol) by spectral data including MS, ^{1}H-, and ^{13}C-NMR. The IC_{50} values of emodin, emodin 3-methyl ether, ω-hydroxyemodin, and *trans*-resvertrol were 2.81, 74.07, 10.49, and 8.77 μM, respectively. They did not inhibit other glycosidase such as glucosidase, mannosidase, and galactosidase, indicating that they were relatively specific inhibitors of neuraminidase.

Key words: Neuraminidase inhibitor, Influenza, Emodin, Emodin 3-methyl ether, Physcion, ω-Hydroxy emodin, *trans*-Resveratrol, *Reynoutria elliptica*

INTRODUCTION

Influenza type A and B viruses cause serious, widespread respiratory infection in humans. Primary infection can lead to a number of complications and secondary infections, particularly in the elderly, those with preexisting airways disease, and many other high-risk groups. As a result, influenza infection is associated with serious morbidity, mortality, and financial burden (Colman, 1993)

Influenza is an enveloped virus and two glycoprotein are displayed on the viral envelope, a haemagglutinin and a neuraminidase (sialidase, NA), (Colman, 1995). The receptor for influenza viruses is a carbohydrate. Sialic acid (*N*-acetyl neuraminic acid, Neu5Ac) is the critical sugar residue which interacts weakly with the viral haemagglutinin to cause attachment of the virus to target cells. After infection and replication, progeny virions bud at the plasma membrane of the infected cell (Willey and Skehe, 1987). Neuraminidase (EC 3.2.1.18) is a surface glycoprotein that possesses enzymatic activity essential for viral replication in both influenza A and B viruses. This enzyme is responsible for catalyzing the cleavage of the α(2-6)- or α(2-3)-ketosidic linkage that exists between a terminal sialic acid and an adjacent sugar residue (Gottschalk, 1957). The breaking of this bond has several important effects. First, it allows for the release of virus from infected cells. Second, it prevents the formation of viral aggregates after release from host cells. Third, this enzyme, by cleaving the sialic acid found in respiratory tract mucins, may prevent viral inactivation and promote viral penetration into respiratory epithelial cells (Colman, 1994; Klenk and Rott, 1988; Palese *et al.*, 1974; Lin *et al.*, 1995; Palese and Compans, 1976). Thus, effective neuraminidase inhibitors can be used for preventing and curing influenza infections.

These backgrounds led us to screen neuraminidase inhibitors from natural products. Out of 260 species of oriental crude drugs, the ethyl acetate soluble fraction of *Reynoutria elliptica* exhibited the highest inhibitory activity against NA. In this report, purification, structure determination, and inhibitory activity of the active compounds will be discussed.

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MATERIALS AND METHODS

General

Herbal extracts were obtained from Korea Research Institute of Chemical Technology, Daejon, Korea. *Reynoutria elliptica* was purchased from the market place located in Daegu, Korea. Fluorescence was measured with Shimadzu RF-5301 (Japan) spectrofluorophotometer. The image analyzer was purchased from Bio-profil (France) and ^{1}H- and ^{13}C-NMR spectra were recorded on an Anvance Digital 400, Bruker. Chemical shifts were given in δ (ppm) from TMS. EIMS was measured on a Shimadzu QP-1000A (Japan) at 70 eV. Silica gel (Kieselgel 60, Art. 7734) and pre-coated TLC plates (Kieselgel 60 F254, Art. 5715 and Art. 1.15685) were from Merck. The Sephadex LH-20 was purchased from Sigma.

Enzyme assay

For screening, the final concentration of methanolic extract of herbal drugs was adjusted to 1 ppm.

Neuraminidase assay using spectrofluorophotometer: Neuraminidase (NA) activity was determined using the method described elsewhere (Myers *et al.*, 1980) with some modification. Briefly, a mixture of 10 μL enzyme (2.5 $\times 10^{-3}$ U, from *Clostridium perfringens*, Sigma), 340 μL 0.04 M sodium acetate buffer (pH 5.0), 10 μL sample solution in MeOH, and 40 μL 0.125 mM substrate [2'-(4-methyl-umbeliferyl)-α-D-*N*-acetylneuraminic acid, Sigma] was incubated for 10 min at 37°C. After the reaction was stopped by adding 3.5 mL of 0.1 M glycine-NaOH buffer (pH 10.4), the fluorescence of reactant (A) was measured at Ex. 360 nm/Em. 440 nm. The control (C) was made by adding MeOH instead of the sample solution. The fluorescence of sample (B) was measured to correct the fluorescence of the sample itself. The percent inhibition was calculated by the following equation.

Inhibition (%)=[A410 of the control (C) (A-B)/A410 of the control (C)]×100.

Neuraminidase assay using image analyzer: The simple neuraminidase assay system was develop-ed as follows. As promptly as four samples having different inhibitory activity were measured by Myers' method, 250 μL of the reaction mixture was taken and its fluorescence under 365 nm was measured with image analyzer. The calibration curve was made by the function between the inhibition ratio from Myers' method and the strength of fluorescence under the image analyzer.

For the screening, mixtures of 10 μL enzyme (2.5×10^{-3} U), 8 μL sample in MeOH, 8 μL of 0.125 mM 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid, and 54 μL 0.04 M sodium acetate buffer (pH 5.0) were incubated in a 96 well-plate for 10 min at 37°C, and then 700 μL 0.1 M glycine-NaOH buffer (pH 10.4) was added to stop the reaction. Two hundred and fifty μL of the reaction mixture was taken to measure the fluorescence under 365 nm and the strength of fluorescence was measured with the image analyzer. The inhibition percent of the screening sample was calculated by the above calibration equation.

Other glycosidase: Glucosidase (from almond, Sigma), galactosidase (from bovine liver, Sigma), and mannosidase (from snail, Sigma) activity were measured according to the method described in the Sigma catalog (2002-2003, Sigma-Aldrich) using *o*-nitrophenyl-β-D-glucopyranoside, *o*-nitro-phenyl-β-D-galactopyranoside, and *p*-nitrophenyl-β-D-mannopyranoside as substrates, respectively.

Extraction and Isolation

Dried *R. elliptica* (1 kg) was refluxed in 5 L MeOH and the extract was evaporated to dryness. The MeOH extract (241.4 g) was suspended in water and the suspension was partitioned with CH_2Cl_2 and EtOAc, consecutively. The EtOAc extract (10.0 g) was chromatographed on a silica gel column (8.5×64 cm, CH_2Cl_2-MeOH=10:1→100% MeOH) to give Fr. I to XXII. A yellowish powdered compound was obtained from Fr. XII and it was purified by washing repeatedly with methanol (RE1, 2.0 g). The compound RE2 (30.0 mg) was recrystallyzed in a mixture of dichloromethane and methanol from Fr. III. The Sephadex LH-20 column (2.8×43 cm, 50→100% MeOH) of Fr. XII afforded RE3 (50.0 mg). Sephadex LH-20 column (2.8×52 cm, 50→100% MeOH) chromatography of Fr. XV afforded RE4 (30.0 mg).

RE1 [emodin(1,3,8-trihydroxy-6-methylanthraquione)]

Orange needles; $FeCl_3$ positive; $C_{15}H_{10}O_5$ (M.W. 270); EIMS *m/z*: 270 [M^+], ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.03 (1H, s, -OH), 11.95 (1H, s, -OH), 7.40 (1H, s, H-4), 7.09 (1H, s, H-2), 7.05 (1H, d, *J*=2.3 Hz, H-5), 6.55 (1H, d, *J*=2.3 Hz, H-7), 2.38 (3H, s, CH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: Table II.

RE2 [emodin 3-methyl ether, physcion (1,8-dihydroxy-3-methoxy-6-methylanthraquinone)]

Red brick needles; $FeCl_3$ positive; $C_{16}H_{12}O_5$ (M.W. 284); EIMS *m/z*: 284 [M^+]; ^{1}H-NMR (400 MHz, chloroform-*d*) δ: 12.20 (1H, s, -OH), 11.99 (1H, s, -OH), 7.60 (1H, s, H-4), 7.33 (1H, s, H-2), 7.06 (1H, d, *J*=2.5 Hz, H-5), 6.66 (1H, d, *J*=2.5 Hz, H-7), 3.94 (3H, s, $-OCH_3$), 2.43 (3H, s, CH_3); ^{13}C-NMR (100 MHz, chloroform-*d*) δ: Table II.

RE3 [ω-hydroxy emodin (1,3,8-trihydroxy-6-hydroxymethylanthraquinone)]

Amorphous yellow powder; Positive to $FeCl_3$; $C_{15}H_{10}O_6$

(M.W. 286); EIMS *m/z*: 286 [M^{+}+1]; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.11 (1H, s, -OH), 12.08 (1H, s, -OH), 10.31 (1H, brs, 3-OH), 7.65 (1H, s, H-4), 7.26 (1H, s, H-2), 7.15 (1H, d, *J*=2.2 Hz, H-5), 6.62 (1H, d, *J*=2.2 Hz, H-7), 5.59 (1H, brs, -CH$_2$O<u>H</u>), 4.61 (2H, s, -C<u>H</u>$_2$OH); ^{13}C-NMR (100-MHz, DMSO-d_6) δ: Table II.

RE4 *trans*-resveratrol (3,5,4'-trihydroxystilbene)]

Colorless needles; Positive to $FeCl_3$; $C_{14}H_{12}O_3$ (M.w. 228); EIMS *m/z*: 228 [M^{+}]; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.57 (1H, s, 4'-OH), 9.22 (2H, s, 3,5-OH), 7.41 (2H, d, *J*=8.5 Hz, H-2'), 6.96 (1H, d, *J*=16.3 Hz, H-7'), 6.84 (1H, d, *J*=16.3 Hz, H-7), 6.77 (2H, d, *J*=8.5 Hz, H-3'), 6.40 (2H, d, *J*=2.1 Hz, H-2), 6.13 (1H, d, *J*=2.1 Hz, H-4); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 139.60 (C-1), 104.62 (C-2,6), 158.83 (C-3,5), 102.07 (C-4), 125.96 (C-7), 128.39 (C-1'), 128.20 (C-2',6',7'), 115.84 (C-3',5'), 157.54 (C-4').

RESULTS AND DISCUSSION

The Myers' method was incongruent for screening a large quantity of samples due to the necessity of a relatively large reaction volume and a complicated protocol. The produced fluorescence of the reaction mixture after enzymatic reaction could be quantitatively measured by the image analyzer. The strength of fluorescence under UV 365 nm was negatively proportional to the inhibitory activity (Fig. 1). Based on this principle, the inhibitory activity of four arbitrary samples was measured according to Myers' method, and then the aliquots were instantly taken to measure the response on the image analyzer. The calibration curves were made by the function between two results [r^2=0.9912, y=(-) 476.2x+70,225, where y was the

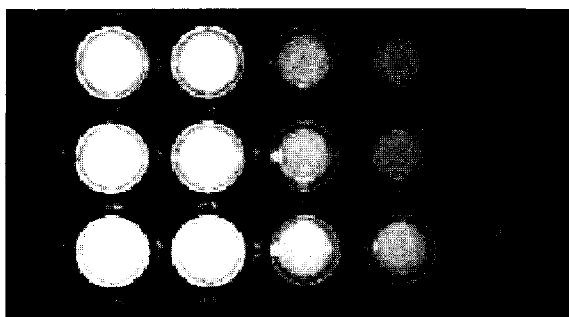

Fig. 1. Measurement of neuraminidase activity by image analyzer. Ten μL of methanol was used as a control and each methanol extract (final concentration 5 ppm) was added to the enzyme reaction mixture. After incubating at 37°C for 10 min., the intensity of fluorescence under UV 365 nm was measured by image analyzer and it was converted into the inhibition percent by the calibration equation in Fig. 2. From the left lane, control (0% of inhibition), methanol extract of *Pseudocolus schellenbergiae* (10% of inhibition), *Magnoliae Flos* (48% of inhibition), *Cladophora wrightiana* (68% of inhibition), and *Sargassum horneri* (89% of inhibition).

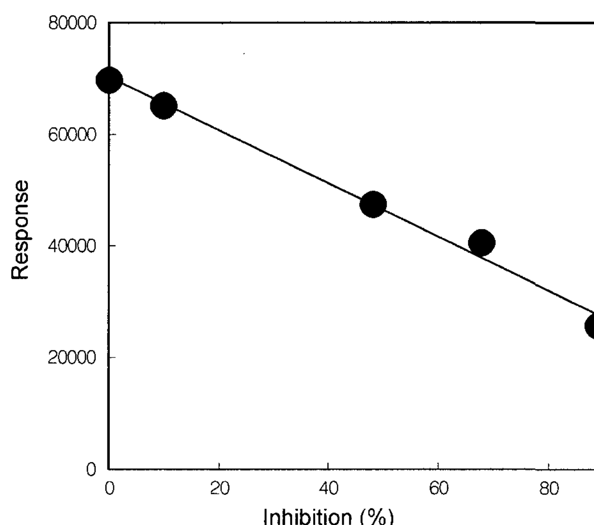

Fig. 2. Calibration curve for neuraminidase activity. The calibration curve was made by taking the inhibition ratio of each sample and the response on image analyzer (the intensity of fluorescence under UV 365 nm) as functions of x and y axis, respectively.

response of the image analyzer and x was the inhibition %] (Fig. 2). The difference of the two methods in inhibition % did not exceed ±3% (data not shown). By this new assay protocol, 260 methanolic extract of crude drugs were tested and out of them, *Terminaria chebula*, *Pulsatilla ko-reana*, *Areca catechu*, *Uncaria gambir*, *Nelumbo nucifera*, *Akebia quinata*, *Uncaria sinensis*, *Rheum undulatum*, *Ammomum tsao-ko*, *Alpinia katsumadai*, and *Reynoutria elliptica* showed more than 90% of inhibition (Table I). The methanolic extract of the above samples were partitioned with EtOAc and their activities were tested. As a result, EtOAc soluble fraction of *R. elliptica* showed the highest activity (98.0% at 1 ppm, data not shown). The activity-guided purification of the EtOAc soluble fraction of *R. elliptica* afforded four inhibitors, RE1, RE2, RE3, and RE4.

RE1 was obtained as orange needles, and positive to $FeCl_3$. The molecular weight was determined as 270 from EIMS spectrum. In ^{1}H-NMR spectrum, two broad aromatic singlets appeared at δ 7.40 (1H) and 7.09 (1H), which could be assigned as the *meta*-coupled protons of an anthraquinone backbone. Two additional *meta*-coupled protons were observed at δ 7.05 (1H, d, *J*=2.3 Hz) and 6.56 (1H, d, *J*=2.3 Hz). In addition, the signal at δ 2.38 showed the typical resonances of a methyl group attached to an aromatic ring. Two hydrogen-bonded hydroxyl protons appeared at δ 12.03 and 11.95. Two α, β-unsaturated ketones (δ 181.08 and 189.47) and twelve aromatic sp^2 carbons were detected in the ^{13}C-NMR spectrum. The structure of RE1 was finally identified as emodin by comparing its NMR data with those in the reported reference (Francis *et al.*, 1998).

Table I. Inhibitory activity of herbal extracts against neuraminidase

Scientific Name	Inhibition (%)	Scientific Name	Inhibition (%)
Acanthopanax sessiliflorum	12	*Asiasarum sieboldi*	3
Acanthopanax sessiliflorum	22	*Aster tataricus*	30
Acontium carmichaelia	8	*Atractylodes japonica*	7
Aconitum carmichaelib	20	*Atractylodes japonica (Lanceae)*	1
Aconitum ciliare	5	*Astragalus membranaceus*	3
Acorus gramineus	28	*Belamcanda chinensis*	23
Acyranthes japonica	3	*Benincasa hispida*	23
Adenophora triphylla	4	*Biota orientalis*	8
Adenophora remotiflorus	21	*Bombyx mori(Batryticatus)*	9
Agastache rugosa	5	*Bombyx mori(Faeces)*	27
Akebia quinata (Lignum)	21	*Boswellia carterii*	33
Akebia quinata (Fructus)	96	*Brassica juncea*	36
Albizzia julibrissin	30	*Broussonetia kazinoki*	50
Alisma orientale	13	*Buddleia officinalis*	26
Aloe ferox	21	*Bupleurum falcatum*	9
Alpinia katsumadai	92	*Caesalpinia sappan*	59
Alpinia officinarum	11	*Carpesium abrotanoides*	47
Alpinia oxyphylla	34	*Carthamus tinctorius*	3
Amomum cardamomum	19	*Cassia tora*	6
Amomum tsao-ko	93	*Caragana sinica*	15
Amomum xanthioides	30	*Celosia argentea*	31
Ampelopsis japnica	15	*Chaenomeles sinensis*	51
Anemarrhena asphodeloides	6	*Chrysanthemum indicum*	6
Angelica dahurica	23	*Chrysanthemum sibiricum*	5
Angelica gigas	2	*Cibotium barometz*	27
Angelica koreana	9	*Cimicifuga heracleifolia*	21
Angelica tenuissima	11	*Cinnamomum cassiac*	36
Anethum graveolens	28	*Cinnamomum cassiad*	35
Anthriscus sylvestris	34	*Cinnamomum cassiae*	26
Aralia cordata	30	*Circium japonicum*	6
Arctium lappa	13	*Cistanche deserticola*	19
Areca catechu (Pericarpium)	31	*Citrus aurantium*	9
Areca catechu (Semen)	92	*Citrus unshiuf*	19
Arisaema amurense	18	*Citrus unshiug*	3
Aristolochia contorta	25	*Clematis mandshurica*	30
Artemisia asiatica	2	*Cnidium officinaleh*	1
Artemisia capillaris	8	*Cnidium officinalei*	3
Asparagus cochinchinensis	6	*Codonopsis pilosula*	26
Coix lachryma-jobi	4	*Ferula assafoetida*	21
Commiphora molmol	30	*Foeniculum vulgare*	1
Coptis japonica	3	*Forsythia viridissima*	3
Cornus officinalis	13	*Fritillaria thunbergii*	2
Corydalis ternata	15	*Gastrodia elata*	2
Crataegus pinnatifida	43	*Gardenia jasminodes*	14
Croton tiglium	43	*Gentiana macrophylla*	8
Cudrania tricuspidata	1	*Gentiana scabra*	20

Table I Continued

Scientific Name	Inhibition (%)	Scientific Name	Inhibition (%)
Curculigo orchioides	39	*Geranium thunbergii*	89
Curcuma longaj	22	*Ginkgo biloba*	7
Curcuma longak	24	*Gleditsia japonica(Spina)*	85
Curcuma zedoaria	19	*Gleditsia japonica(Fructus)*	2
Cucumis melo	12	*Glycyrrhiza glabra*	23
Cuscusta chinensis	34	*Hovenia dulcis*	4
Cynanchum atratum	4	*Hordeum vulgare*	11
Cynomorium songaricum	44	*Houttuynia cordata*	5
Cyperus rotundus	12	*Hydnocarpus anthelmintica*	31
Daphne genkwa	24	*Imperata cylindrica*	22
Dendrobium nobile	61	*Inula japonica*	30
Dianthus chinensis	3	*Isatis tinctoria*	21
Dictamus albus	2	*Juncus effusus*	19
Dioscorea japonica	6	*Kalpnax pictus*	11
Dioscorea tokoro	18	*Kochia scoparia*	1
Diospyros kaki	45	*Ledebouriella seseloides*	23
Dolichos lablab	16	*Leonurus sibiricus*	12
Draba nemorosa	39	*Ligustrum lucidum*	17
Drynaria fortunei	3	*Lilium lancifolium*	11
Dryobalanops aromatica	39	*Lindera strychnifolia*	45
Echinops setifer	20	*Liriope platyphylla*	8
Eclipta prostrata	27	*Lithospermum erythrorhizon*	60
Ephedra sinica	23	*Lonicera japonica(Flos)*	11
Epimedium koreanum	17	*Lonicera japonica(Caulis et Folium)*	44
Eriobotrya japonica	33	*Loranthus parasiticus*	0
Erucibe obtusifolia	10	*Lycium chinense(Radicis Cotex)*	16
Eucommia ulmoides	2	*Lycium chinense(Fructus)*	8
Eugenia caryophyllata	35	*Lycopus coreanus*	19
Euonymus alatus	58	*Lygodium japonica*	26
Euphoria longana	33	*Magnolia denudata*	22
Euryale ferox	52	*Magnolia officinalis*	65
Evodia officinalis	25	*Malva verticillata*	22
Melia azedarach	6	*Polygonum aviculare*	38
Mentha arvensis	26	*Polygonum multiflorum*	2
Momordica cochinchinensis	22	*Polyporus umbellatus*	34
Morinda officinalis	11	*Poncirus trifoliata*	18
Morus alba(Cortex)	22	*Poria cocos*	41
Morus alba(Fructus)	16	*Puearia thunbergii(Radix)*	28
Morus alba(Folium)	36	*Puearia thunbergii(Flos)*	34
Mucuna birdwoodiana	85	*Prunella vulgaris*	6
Myristica fragrans	48	*Prunus armeniaca*	16
Nardostachys chinensis	38	*Prunus mume*	4
Nelumbo nucifera	97	*Prunus nakaii*	10
Nepeta japonica	9	*Prunus persica*	22
Omphalia lapidescens	26	*Psoralea corylifolia*	18
Pachyma hoelen	8	*Pulsatilla koreana*	92

Table I. Continued

Scientific Name	Inhibition (%)	Scientific Name	Inhibition (%)
Paeonia albiflora	33	*Pyrrosia lingua*	28
Paeonia japonica	42	*Quisqualis indica*	27
Paeonia moutan	40	*Raphanus sativus*	14
Paeonia obovata	27	*Reynoutria elliptica*	90
Patrinia villosa	22	*Rheum palmatum*	45
Perilla sikokiana(Folium)	50	*Rheum undulatum*	93
Perilla sikokiana(Semen)	4	*Rhus javanica*	83
Persicaria tinctoria	3	*Ricinus communis*	23
Pharbitis nil	5	*Rosa laevigata*	35
Phellodendron amurense	4	*Rubus coreanus*	73
Phlomis umbrosa	14	*Rubia alkane*	4
Phragmites communis	6	*Sanguisorba officinalis*	33
Phyllostachys bambusoides	3	*Santalum album*	20
Phyllostachys nigra	3	*Saururus chiensis*	11
Phytolaca esculenta	2	*Saussurea lappa*	28
Pinellia ternata	28	*Schizandra chinensis*	18
Pinus densiflora	44	*Scripus flaviatilis*	18
Pinus densiflora	12	*Scrophularia buergeriana*	1
Piper nigrum	4	*Scutellaria baicalensis*	12
Piper longum	20	*Siegesbeckia pubescens*	7
Plantago asiatica	22	*Sinomenium acutum*	19
Platycodon grandiflorum	2	*Slavia miltiorrhiza*	58
Polygala tenuifolia	32	*Smilax China*	88
Polygonatum odoratum	18	*Sophora flavescens*	5
Polygonatum sibiricum	7	*Sophora japonica*	31
Sophora subprostrata	22	*Triticum aestivum*	18
Spirodela polyrhiza	22	*Tussilago farfar*	8
Stemona japonica	6	*Typha orientalis*	28
Strychnos ignatii	26	*Ulmus macrocarpa*	13
Taraxacum platycarpa	3	*Uncaria gambir*	96
Terminaria chebula	91	*Uncaria sinensis*	93
Thuja orientalis	1	*Vitex rotundifolia*	7
Torilis japonica	1	*Xanthium strumarium*	3
Tribulus terrestris	4	*Zanthoxylum piperitum*	15
Trichosanthes kirilowiil	2	*Zea mays*	14
Trichosanthes kirilowii(Radix)	7	*Zingiber officinale*	3
Trichosanthes kirilowii(Semen)	26	*Zizyphus jujuba*	31
Trigonella foenum-graecum	12	*Zizyphus vulgares*	29

The Korean traditional names of the crude drugs are [a]Buja, [b]Odu, [c]Yookgye, [d]Gyeji, [E]gyepi, [f]Jinpi, [g]Chungpi, [h]Tocheongung, [i]Cheongung, [j]Ganghwang, [k]Ulgeum, and [l]Cheonhwabun. The final concent·ation of plant extract is 1 ppm.

RE2 was positive to $FeCl_3$ and showed [M^+] at *m/z* 284 in the EIMS spectrum. The ^{1}H-NMR data were very similar to those of RE1 except for an additional resonance at δ 3.94 (3H, s), which suggested that RE2 was a methylated compound of RE1. In ^{13}C-NMR spectrum, two ketone signals (δ 192.03 and 181.33), twelve aromatic carbons, one methoxyl, and a methyl signal (δ 21.92) were detected. The methyoxylated position was postulated to be C-3 since two hydrogen-bonded protons were observed at δ 12.20 and 11.99, which could be assigned

to hydroxyl groups at C-1 and C-8. In addition, the *Rf* values [0.51, *n*-hexane-EtOAc (7:1) on normal phase; 0.40, 95% MeOH on RP-18] and ^{1}H-NMR data of methylated RE1 were identical with those of RE2. The methylation of RE1 (emodin) was performed by diazomethane in an ice bath, and under this mild condition, hydrogen-bonded hydroxyl groups rarely undergo methylation. The structure of RE2 was finally identified as emodin 3-methyl ether (physcion) by referring to documented data (Ko *et al.*, 1995).

RE3 was also positive to $FeCl_3$. The molecular ion peak was detected at *m/z* 286 in the EIMS spectrum. Aromatic protons at δ 6.62, 7.15, 7.26, and 7.65 suggested RE3 was also an analogue of emodin. A methylene proton (δ 4.61) and an alcoholic hydroxyl resonance (δ 5.59) were detected, suggesting the presence of hydroxymethyl moiety. Two hydrogen bonded hydroxyl protons were detected at δ 12.03 and 11.95 with a broad signal at δ 10.03 in the ^{1}H-NMR spectrum. RE3 was finally identified as ω-hydroxyemodin by comparing its ^{1}H- and ^{13}C-NMR data with those reported elsewhere (Murakami *et al.*, 1987).

RE4 was positive to $FeCl_3$, indicating that it had phenolic hydroxyl groups. The molecular ion peak was found at *m/z* 228. In ^{1}H-NMR, nine aromatic proton signals were detected at δ 6.13~6.96 with three exchangeable hydroxyl protons at δ 9.22 and 9.87. The configuration of RE4 was determined to be a *trans* from the coupling constants (16.3 Hz, each) between δ 6.84 and 6.96. Fourteen carbon signals including three oxygenated aromatic carbons [δ 157.54 (C-4') and 158.83 (C-3,5)] were observed in the ^{13}C-NMR spectrum. RE4 was postulated to be *trans*-resveratrol from the spectral data, and this was confirmed by comparing its NMR data with those in references (Chen *et al.*, 2001; Likhitwitayawuid *et al.*, 2000).

The structures are presented in Fig. 3 and the ^{13}C-NMR data are listed in Table II.

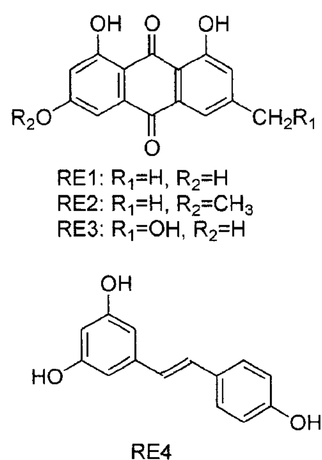

Fig. 3. *Structures of RE1, RE2, RE3, and RE4, RE1; emodin, RE2; emodin 3-methyl ether (physcion), RE3; (ω-hydroxy emodin), RE4; trans-resveratrol.*

All compounds inhibited neuraminidase in a dose-dependent manner (Fig. 4). The IC_{50} values of RE1, 2, 3, and 4 were 2.81, 74.07, 10.49, and 8.77 μM, respectively. To check the enzyme specificity, the inhibitory activities on other glycosidase such as glucosidase, galactosidase, and mannosidase were compared with that of neuraminidse. Up to 5 ppm of the isolated compounds inhibited only less than 10% of above enzyme activities (Table III). Thus, they were thought to be relatively specific inhibitors of neuraminidase.

Table II. ^{13}C-NMR data of **RE1**, **2**, and **3**

No.	RE1[a]	RE2[a,b]	RE3[a]
1	161.27	161.82	161.36
2	123.94	119.54	120.69
3	148.06	149.19	152.78
4	120.30	120.81	116.98
5	108.66	108.03	108.74
6	165.49	164.77	165.54
7	107.75	106.50	107.84
8	164.32	162.09	164.38
9	189.47	192.04	189.66
10	181.08	181.29	181.33
11	134.85	137.17	135.08
12	108.70	113.73	108.92
13	113.12	115.82	114.02
14	132.56	133.45	132.86
15	21.39	21.98	61.91
OCH_3		56.33	

[a]δ ppm from TMS. Recorded in DMSO-d_6. [b]Recorded in chloroform-*d*

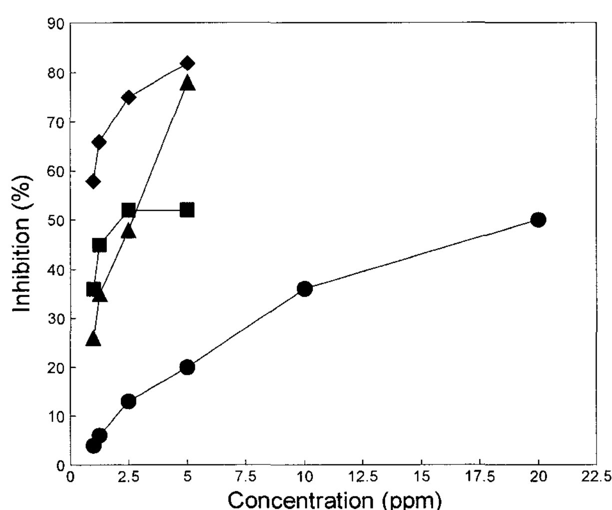

Fig. 4. *Concentration-dependant inhibition of neuraminidase by isolated compounds. Legends: -◆-; RE1 (emodin), -●-; RE2 (emodin 3-methyl ether), -▲-; RE3 (ω-hydroxy emodin), -■-; RE4 (trans-resveratrol).*

Table III. Inhibitory activity against other glycosidase

Enzyme	RE 1		RE 2		RE 3		RE 4	
	5[a]	20	5	20	5	20	5	20
Glucosidase	0.1[b]	0.2	0.2	0.5	0.1	0.3	0.0	0.0
Galactosidase	0.0	0.1	2.0	10.0	1.0	3.1	0.1	3.8
Mannosidase	0.1	0.2	0.1	0.3	0.0	0.2	0.0	0.1
Neuraminidase	20.0	49.8	82.4	ND[c]	77.8	ND	52.3	ND

[a]Ppm. [b]Presented in %. [c]Not determined.

Many neuraminidase inhibitors having Neu5Ac2en (2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid) skeleton have been developed (Von Itzstein *et al.*, 1993; Burmeister *et al.*, 1992; Varghee *et al.*, 1992); however, natural inhibitors have rarely been studied. It is interesting that emodin 3-methyl ether is about ten times less active than ω-hydroxy emodin and emodin even though they are very similar in structure each other, suggesting that 3-hydroxyl group might be important for the stronger activity. It is necessary to investigate much more numbers of anthraquinones for establishing fundamental structure-activity relationship among them. The anthraquinones, which were firstly isolated in this study as a neuraminidase inhibitor, are expected to be useful in preventing and curing influenza.

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REFERENCES

Burmeister, W. P., Ruigrok, R. W., and Cusack, S., The 2.2 resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J.*, 11, 49-56 (1992).

Chen, L., Han, Y., Yang, F., and Zhang, T., High-speed counter-current chromatography separation and purification resvertrol and piceid *Polygonum cuspidatum*. *J. Chromatogr.* A., 907, 343-346 (2001).

Colman, P. M., Influenza virus neuraminidase: structure, antibiotics and inhibitors. *Protein Sci.*, 3, 1687-1696 (1994).

Colman, P. M., Design and antiviral properties of influenza virus neuraminidase inhibitors. *Pure Appl. Chem.*, 67, 1683-1688 (1995).

Colman, P. M., A novel approach to antiviral to therapy for influenza. *J. Antimicrob. Chemother.*, 44, 17-22 (1999).

Francis, G. W., Aksnes, D. W., and Holt, Q., Assignment of the ^{1}H and ^{13}C NMR spectra of anthraquinone glycoside from *Rhamnus frangula*. *Mag. Res. Chem.*, 36, 769-772 (1998).

Gottschalk, A., The specific enzyme of influenza virus and *Vibrio cholerae*. *Biochem. Biophys. Acta.*, 23, 645-646 (1957).

Klenk, H. O. and Rott, R., The molecular biology of influenza virus pathogenicity. *Adv. Virus Res.*, 34, 247-280 (1988).

Ko, S. K., Whang, W. K., and Kim, I. H., Anthraquinone and stilbene derivatives from the cultivated Korean Rhubarb Rhizomes. *Arch. Pharm. Res.*, 18, 282-288 (1995).

Lin, C., Eichelberger, M. C., Compans, R. W., and Air, G. M., Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly or budding. *J. Virol.*, 69, 1099-1106 (1995).

Likhitwitayawuid, K., Sritularak, B., and De-Eknamkul, W., Tyrosinase inhibitors from *Artocarpus gomezianus*. *Planta Medica*, 66, 275-277 (2000).

Murakami, H., Kobayashi, J., Musuda, T., Morooka, N., and Ueno, Y., ω-Hydroxyemodin, a major hepatic metabolite of emodin in various animals and its mutagenic activity. *Mutation Res.*, 180, 147-153 (1987).

Myers, R. W., Lee, R. T., Lee, Y. C., and Thomas, G. H., The synthesis of 4-methylumberiferyl α-ketoside of *N*-acetylneuraminic acid and its use in a fluorometric assay for neuraminidase. *Anal. Biochem.*, 101, 166-174 (1980).

Palese, P. and Compans, R. W., Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-*N*-trifluoroacetyl neuraminic acid (FANA): mechanism of action. *J. General Virol.*, 33, 159-163 (1976).

Palese, P., Tabita, U., Ueda, M., and Compans, R. W., Characterization of temperature sensitive influenza virus mutants. *Virology*, 61, 397-410 (1974).

Varghee, J. N., Mckimm-Breschkin, J. L., Caldwell, J. B., Kortt, A. A., and Colman, P. M., The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. *Protein*, 14, 327-332 (1992).

von Itzstein, N., Kok, G. B., and Pegg, M. S., Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature*, 363, 418-423 (1993).

Willey, D. C. and Skehel, J. J., The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.*, 56, 365-394 (1987).