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RESEARCH PAPER

Inhibition Effect of Flavonoid Compounds against Neuraminidase Expressed in *Pichia pastoris*

Thi Thanh Hanh Nguyen, Hee-Kyoung Kang, Young-Min Kim, Tae-Su Jang, and Doman Kim

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Abstract Neuraminidase (NA) is one of the two glycoproteins on the surface of influenza virus, which cleaves terminal sialic acid residues and facilitates the release of virions from infected cells. The recombinant NA from H5N1 influenza virus strain A/Vietnam/1203/04 was expressed in *Pichia pastoris* X33 as a 45 kDa protein that displayed a K_m of 9.96 ± 1.26 μM with fluorogenic substrate, 2'-(4-methylumbelliferyl)-α-D-*N*-acetyl neuraminic acid. Partially purified NA was used for the inhibition and kinetic assays with eight flavonoid compounds and gallic acid. Among them, gallocatechin gallate (GCG) showed the best inhibition against NA with the IC_{50} of 8.98 ± 0.46 μM and showed a competitive inhibition pattern with K_i value of 8.34 ± 0.25 μM. In molecular docking experiments, GCG displayed a binding energy of -13.71 kcal/mol to the active site of NA and the galloyl moiety was required for NA inhibition activity.

Keywords: neuraminidase, H5N1, *Pichia pastoris*, flavonoid, molecular docking, catechin

Thi Thanh Hanh Nguyen, Hee-Kyoung Kang, Doman Kim*
Department of Biotechnology and Bioengineering, and Research Institute for Catalysis, College of Engineering, Chonnam National University, Gwangju 500-757, Korea
Tel: +82-62-530-1844; Fax: +82-62-530-1949
E-mail: dmkim@jnu.ac.kr

Young-Min Kim
Infection Control Material Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeonbuk 580-185, Korea

Tae-Su Jang, Doman Kim
Research Institute of Bio Food Industry, The Green Bio Research Complex, Seoul National University, Pyeongchang 232-916, Korea

1. Introduction

Influenza A viruses caused annual outbreaks in humans and domestic animals. Three major influenza A pandemics were recorded during the 20th century: the 1918-1919 "Spanish flu" representing the H1N1 influenza virus, the 1957-1958 "Asian flu" (H2N2), and the 1968–1969 "Hong Kong flu" (H3N2) [1-3]. The 1918-1919 pandemic caused the highest number of casualties: more than 500,000 people died in the USA, and more than 50 million people died worldwide [3]. The "Asian flu" in 1957-1958 caused about 70,000 deaths in the USA, and the "Hong Kong flu" in 1968-1969 caused about 34,000 deaths [1,4]. Since 1997, different subtypes (H5N1, H7N7, and H9N2) of avian influenza viruses have been identified. Among them, the highly pathogenic H5N1 influenza viruses have severely affected to the poultry industry and posed a serious threat to human health, resulting in more than 50% fatality among more than 300 confirmed human cases [5]. The Influenza virus membranes contain two glycoproteins: haemagglutinin (HA) and neuraminidase (NA). HA mediates cell-surface sialic acid receptor binding to initiate virus infection. After virus replication, NA removes sialic acid from virus and cellular glycoproteins to facilitate virus release and the spread of infection to new cell. The distinct antigenic properties of different haemagglutinin and neuraminidase molecules are used to classify influenza type A viruses into subtypes: 16 for HA (H1-H16) and 9 for NA (N1-N9) [2]. Because of the importance of this enzyme in the pathogenesis of influenza virus infection, it has been regarded as a drug target for finding new drugs for the treatment of influenza [5,6]. The avian H5N1 viruses might eventually become capable of species barrier crossing and of effective human to human transmission [1,4].

Flavonoids are a large group of naturally occurring

phenolic compounds ubiquitously distributed in the plant kingdom. Over 4,000 varieties of flavonoids have been identified [7]. Flavonoids can act as potent antioxidants, display anti-inflammatory, antiallergic, antihemorrhagic, antimutagenic, antineoplastic, and hepatoprotective activities [7,8]. There have some reports anti-influenza virus activities by inhibiting NA activity of several flavonoids compounds such as EGCG, EGC, and ECG against neuraminidase from A/H1N1, A/H3N2 and B virus [9]; and 25 flavonoids (apigenin, catechin, epicatechin, luteolin, quercetin, rutin, *etc*) against neuraminidase from A/PR/8/34 (H1N1), A/Jinan/15/90 (H3N2), and B/Jiangshu/10/2003 [10]. However, there has hitherto been no report on the strong inhibition activity of gallocatechin gallate (GCG) against NA from H5N1 influenza virus strain A/Vietnam/1203/04, or on the structure-activity relationship activity among the aforementioned flavonoid compounds against NA from H5N1.

Herein, we report on the *in vitro* inhibition study using compounds belonging to four groups of flavonoids (flavonol, flavanonol, isoflavone, and flavan-3-ol) and gallic acid, against NA expression from *Pichia pastoris* X33 (*P. pastoris* X33) and the structure-activity relationship of them. The detailed mechanism of GCG inhibition was investigated by enzyme kinetic and molecular docking studies.

2. Material and Methods

2.1. Enzyme preparation

The procedures for construction, transformation, and screening for neuraminidase head domain (NA) of H5N1 influenza virus strain A/Vietnam/1203/04 were according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The vector pJ201 bearing gene encoding neuraminidase from H5N1 influenza virus strain A/Vietnam/1203/04 (amino acid residues: 63 – 449, GenBank accession no. AY818141) was constructed and optimized genetic codons based of yeast *P. pastoris* using a custom gene synthesis service (DNA 2.0, Menlo Park, USA). The NA gene was isolated from the vector pJ201 by cutting with *Eco*RI and *Not*I and ligated into the *Eco*RI/*Not*I digested pPICZαA vector (pNA). This construct allows secretion of the neuraminidase into culture medium with in-frame N-terminal α-factor secretion signal and the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag. The resulting construct, pNA, was used to transform *E. coli* DH5α using a standard heat shock method. Transformants harbouring pNA were selected from LB agar low salt medium (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% NaCl, pH 7.5) containing 25 μg/mL Zeocin™. Plasmid pNA was amplified in *E. coli* DH5α and was linearized by *SacI* digestion and transformed into *P. pastoris* X-33 by modified lithium chloride methods [11]. Screening for positive clones was done by PCR method with two sets of primers: set one contained NA primers and set two contained α-factor and 3AOX1 primers. The PCR were conducted by using PCRmix (Bioneer, Daejeon, Korea) with thermal cycling of 95ºC for 5 min, followed by 25 cycles of 95ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min 20 sec with final step of 72ºC for 7 min and the PCR product was analyzed by agarose gel electrophoresis.

For expression, the selected clone was inoculated into 100 mL BMGY (1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% w/v yeast nitrogen base with ammonium sulphate without amino acids, 4×10^{-5}% w/v biotin and 1% v/v glycerol) in a 1 L flask and incubated at 28ºC with shaking at 220 rpm for 16 h. Cells were harvested by centrifugation at 3,000 × *g* for 10 min and resuspended cell pellet to an OD_{600} at 1.0 into 500 mL BMMY media (same components of BMGY except 0.5% absolute methanol instead of glycerol) in 2 of 2 L flasks and incubated at 28ºC with shaking at 220 rpm. To maintain methanol induction of the AOX1 promoter, 0.5% (v/v) methanol was fed every 24 h during the fermentation period. Protein expression of pPICZαA transformed into *P. pastoris* X-33 was conducted as negative control. The yeast cells were then separated from the broth by centrifugation at 8,000 × *g* for 15 min. The pellet was discarded and the supernatant was used for ammonium sulphate fractionation (0 ~ 90%). The fraction that showed enzyme activity was collected, dialyzed against 40 mM Na-P buffer (pH 7.0) and protein band was analyzed using SDS-PAGE and Western Blot.

2.2. Neuraminidase (NA) activity assay

Neuraminidase enzymatic activity assay measured 4-methylumbelliferone (4MU) released from the fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA, Sigma, St. Louis, USA). The reaction contained 0.34 units NA enzyme, 40 mM Na-P buffer (pH 7.0) and 20 μM MUNANA, the enzyme reaction was followed from 0 to 60 min at 37ºC using a fluorescence plate reader. The amount of fluorescent product, 4-methulumbeliferone was measure in a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, USA) with excitation and emission wavelengths of 355 and 460 nm, respectively. A standard curve was generated by plotting relative fluorescence intensity against the amount of free 4MU. One unit of NA was defined as one micro molar of 4MU produced per min at 37ºC. Kinetic parameters of recombinant neuraminidase were obtained using 5, 10, 15, 20, 40, and 60 μM MUNANA in the fluorescent assay at 52 min. Reactions were linear within this period. Michaelis-Menten constant (K_m) was calculated from the Lineweaver-Burk

plot using the SigmaPlot program (SPSS, San Diego, USA).

2.3. *In vitro* assay of NAH potential inhibitor activity

Quercetin, puerarin, epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epicatechin gallate (ECG), and gallic acid were purchased from Sigma; ampelopsin was purchased from ZR chemicals (Shanghi, China). Ampelopsin glucoside was synthesized enzymatically and purified as described previously [12]. GCG, EGCG, EGC, ECG and ampelopsin glucoside were dissolved in water and quercetin, puerarin, gallic acid and ampelopsin were dissolved in DMSO at a stock concentration of 10 mM. Inhibitory activity of each test compound was determined by measurement of the remaining activity of NA at 200 μM of each tested compound. The enzymatic reaction was composed of 0.34 U/mL of enzyme, 10 μM MUNANA, 200 μM of test compound, and 40 mM potassium phosphate buffer (pH 7.0). Reactions were run for 30 min at 37°C by continuous monitoring of fluorescence using a SpectraMax Gemini XPS apparatus with excitation and emission wavelengths of 355 and 460 nm, respectively.

The inhibition was calculated using the equation:

$$\text{Activity left (\%)} = [(SS_0)/(CC_0)]\times 100 \quad (1)$$

Where C is μM 4MU of the control (enzyme, buffer, and substrate) after 30 min of incubation, C_0 is μM 4MU of the control at zero time, S is μM 4MU of the tested samples (enzyme, sample solution, and substrate) after incubation, and S_0 is μM 4MU of the tested samples at zero time. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of inhibitor necessary to reduce NA activity by 50% relative to a reaction mixture containing NA enzyme but no inhibitor.

2.4. Enzyme kinetics

Inhibition kinetic study was performed for GCG which was the best inhibitor against NA. The method was similar to those used in kinetic study of the recombinant enzyme, except for the use of multiple concentrations of the inhibitor (0 ~ 10 μM) and variable concentrations of substrate (5 ~ 10 μM). The type of inhibition was determined using Lineweaver-Burk plots and a Dixon plot (1/v as a function of inhibitor concentration, [I]) and kinetic parameters (K_i) was calculated using the SigmaPlot program.

2.5. Molecular docking study of neuraminidase with GCG

The Autodock 3.0.5 docking software [13] was used to perform the automated molecular docking between NA and GCG. The NA crystal structure [PDB code 2HU0 with inhibitor (3R,4R,5S)-4-(acetylamino)-5-amino-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (G39)] [2] was prepared for docking and post-docking refinement. For docking experiment between GCG and 2HU0, all water molecules and the inhibitor (G39) located in the active site of 2HU0 were removed, and the structure information containing only the amino acid residues of the NA enzyme was used. The process of molecular docking used between GCG and NA has been described previously [14,15]. The hydrogen interaction between GCG and NA active site pocket was visualize by Ligplot software [16].

3. Results and Discussion

3.1. Preparation of recombinant NA

We prepared active NA of H5N1 (CAZy - Glycoside hydrolase family 34) which is different from commercially available NA from *Clostridium perfringens* (CAZy - Glycoside Hydrolase family 33). The 1161 bp gene encoding for 387 amino acids residues (63 ~ 449) of NA from Influenza virus A/Vietnam/1203/2004 H5N1 was cloned into the pPICZáA expression vector (pNA) and the pNA plasmid was linearized by *SacI* digestion that was further incorporated into the AOX1 locus of *P. pastoris*. A single clone was selected for expression; the recombinant NA was resolved electrophoretically as a band of approximately 45 kDa (Fig. 1A). To confirm that the clone secreted NA, Western blotting (Fig. 1B) was performed using the anti-His antibody; NA was apparent as the same band upon SDS-PAGE. To calculate the kinetic parameters

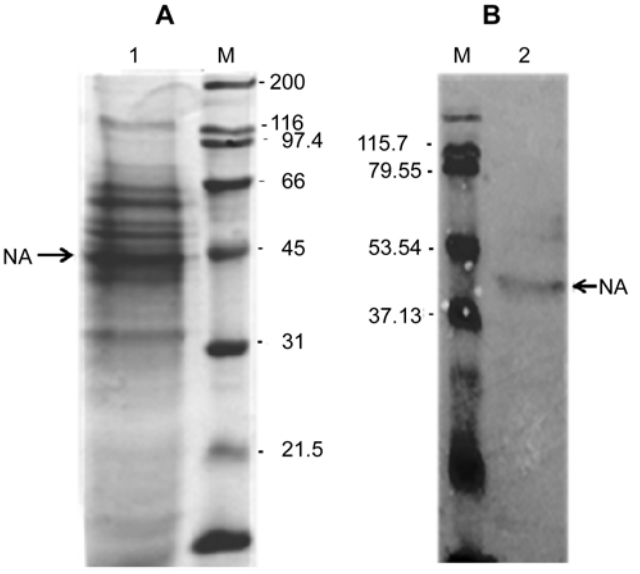


Fig. 1. SDS-PAGE (A) and Western blot (B) results of NA after ammonium sulfate fractionation. Lane M: MW marker; lane 1: SDS-PAGE of active ammonium sulfate fractionation; lane 2: Western blot of active ammonium sulfate fractionation.

of NA, enzyme activity was analyzed with fluorescent substrate ranging from 5 to 60 μM (final concentration). The Linewaever-Burk plot showed linear line interception on the 1/*v* axis. The Michaelis-Menten constant K_m derived from the Lineweaver-Burk plot was 9.96 ± 0.26 μM (Fig. S1).

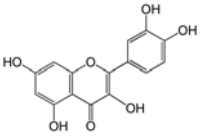
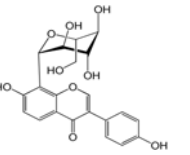
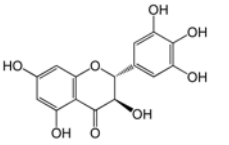
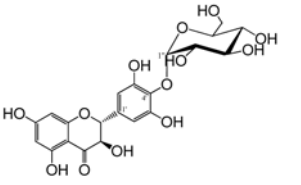
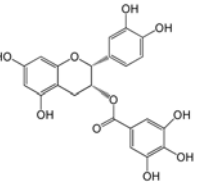
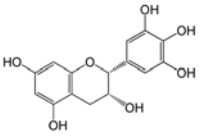
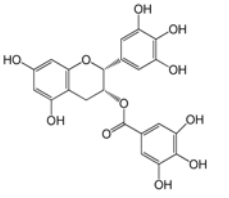
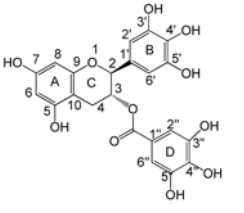
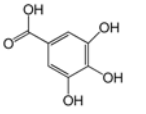
3.2. NA inhibition by flavonoid compounds

The inhibitory activity of gallic acid and eight flavonoid compounds belonging to four groups of flavonoids (flavanonol (ampelopsin, ampelopsin glucoside), flavonol (quercetin), isoflavones (puerarin), and flavan-3-ol (EGC, ECG, EGCG, GCG)) against NA were examined at a concentration of 200 μM. The primarily inhibition activity results were showed in Table 1. EGC and gallic acid inhibited 9.3 and 14.0% of NA activity. Ampelopsin inhibited 27.9% of NA activity while ampelopsin glucoside inhibited 58.2% of NA activity. Quercetin inhibited 35.2% of NA activity. ECG inhibited 36.7% of NA activity, EGCG inhibited 58.0%, and GCG inhibited 87.1% of NA activity. Among the tested compounds, GCG, the best inhibitor against NA with an IC_{50} value of 8.98 ± 0.46 μM, was used to analyze the mode of inhibition. For the analysis of the inhibition mode of GCG, both Lineweaver-Burk and Dixon plots were used. K_i value was calculated from the Dixon plots using the SigmaPlot program. The inhibition of NA by GCG is illustrated in Fig. 2. GCG exhibited competitive inhibition toward NA because the Lineweaver-Burk plot of 1/*v* versus 1/[S] resulted in a family of straight lines with the same y-axis intercept (Fig. 2A). The K_i value of GCG was determined to be 8.34 ± 0.25 μM, from the common x-axis intercept of lines on the corresponding Dixon plot (Fig. 2B).

3.3. Molecular docking between NA and GCG

To further investigate the interaction of GCG with the NA enzyme, molecular docking simulations of the binding of GCG at the NA active site (X-ray crystal structure with PDB code 2HU0) was carried out using Autodock 3.0.5. The free binding energy of GCG was determined to be -13.71 kcal/mol. The binding between GCG and the active site pocket of NA is shown in Fig. 3A. To elucidate the interaction of NA with GCG, the potential hydrophobic and H-bonds interactions between amino acid residues at the active site of NA and GCG were investigated using the Ligplot program. Fig. 3B shows the details of the specific interactions between GCG and NA. Carbon atoms of EGCG interacted hydrophobically with Tyr 347, Arg 371, Trp 403, Ile 427, Arg 430, Pro431, and Thr 439 of NA. GCG formed five hydrogen bonds (H-bond) with residues in the catalytic binding pocket of NA. The O atom of carboxyl group of Asn 325 and Asn 369 accepted H-bond from 5"-OH and 4"-OH galloyl group of ring D with

Table 1. Chemical structure and primarily inhibition assay of flavonoid compounds against NA

Compound	Chemical structure	Primarily inhibition activity (%)*
Quercetin		35.2
Puerarin		57.6
Ampelopsin		27.9
Ampelopsin glucoside		58.2
Epicatechin gallate (ECG)		36.7
Epigallocatechin (EGC)		9.3
Epigallocatechin gallate (EGCG)		58.0
Gallocatechin gallate (GCG)		87.1
Gallic acid		14.0

*Inhibitory activity at 200 μM and triplicate.

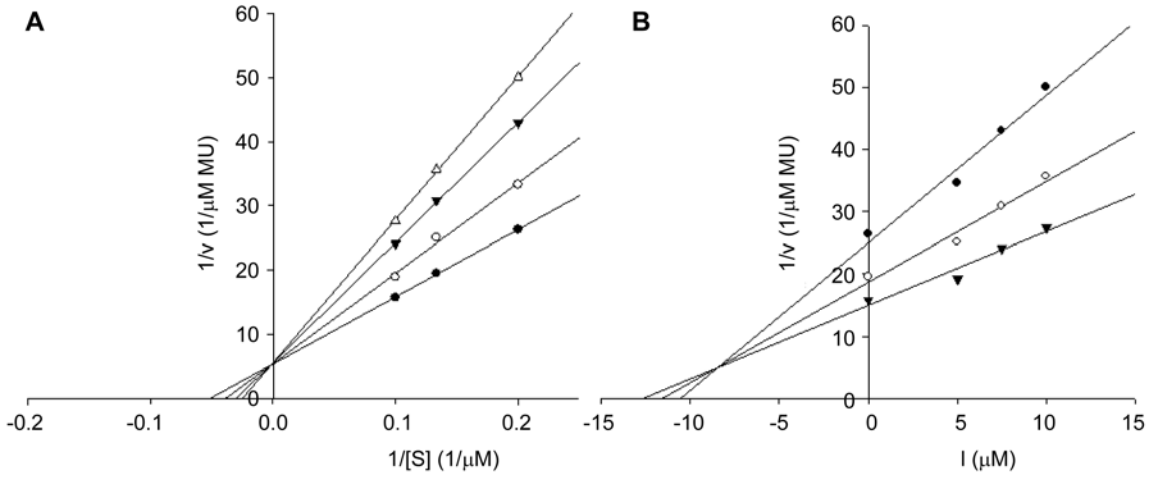


Fig. 2. Lineweaver-Burk plot (A) and Dixon plot (B) of the inhibition against NA by GCG. The kinetic constants, K_i, were calculated using linear regression analysis. A: GCG concentration 0 μM (●), 5 μM (○), 7.5 μM (▼), 10 μM (△). B: MUNA substrate concentrations 5 μM (●), 7.5 μM (○), and 10 μM (▼).

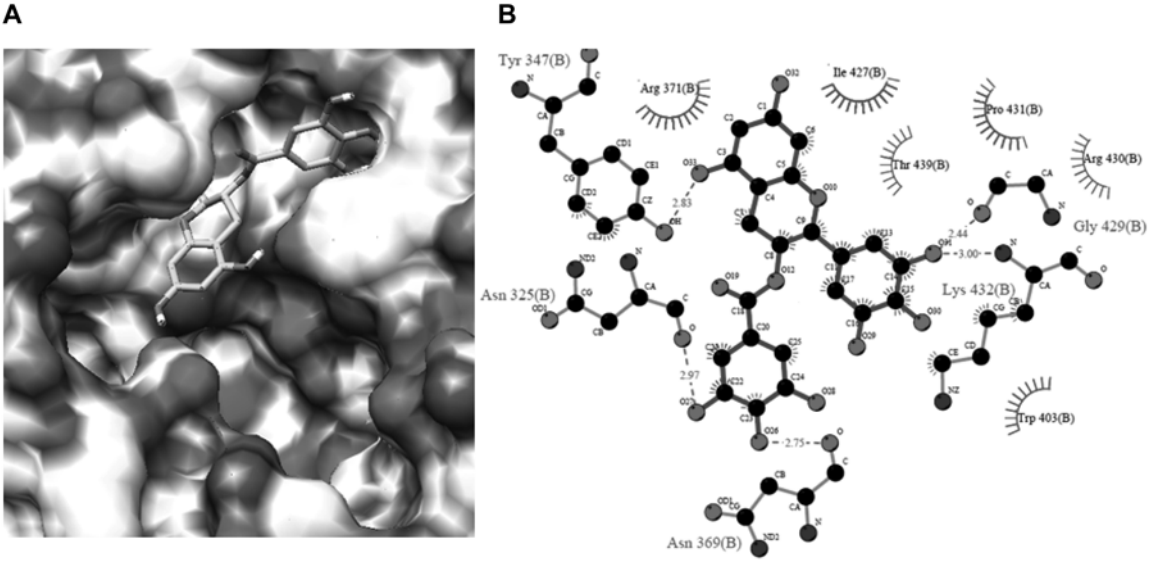


Fig. 3. Computational docking and hydrophobic and hydrogen bond interactions of GCG with amino acid residues in the active site of NA. (A) Comparison of binding modes of GCG (green) in the active site pocket of NA. (B) Hydrophobic and H-bond interactions between GCG and amino acid residues in the active site of NA. H-bond interactions are represented by green dashed lines (red, oxygen; cornflower blue, nitrogen; black, carbon).

distances at 2.97 and 2.75 A°, respectively. The Hydroxyl group of Tyr 347 form H-bonds with 5-OH group of ring A at 2.83 A°. The O atom of 3'-OH group of the ring B have two H-bonds with the O atom of carboxyl group of Gly 429 and N atom of amino group of Lys432 with distant at 2.44 and 3.00 A°, respectively.

3.4. Structural activity relationship of flavonoids compounds against NA

EGC, ECG, ampelopsin, EGCG and GCG contain the same B-ring and the increasing order inhibitory activity was EGC < ampelopsin < EGCG < GCG. EGCG and GCG have a galloy moiety at the 3-OH position, displayed stronger NA inhibitory activity than EGC and ampelopsin. So gallic acid was used for *in vitro* assay, and it showed 14.0% inhibition activity against NA from H5N1 at 200 μM. These results were similar with previous report about potent inhibitors of influenza virus growth including A/H1N1, A/H3N2 and B virus in MDCK cells of EGCG, ECG, EGC and the results showed that galloy group at the 3-OH of catechin skeleton plays an important role on the observed antiviral activity [9]. However, comparison between

EGCG and GCG, GCG (2S, 3R type), which is a C-2 epimeric isomer of EGCG (2R, 3R type) showed 1.5-times higher NA inhibitory activity than EGCG. Puerarin which lack the B-ring, showed higher inhibitory activity compared with ampelopsin and quercetin. Quercetin which lacks the 5'-OH group of B ring and contains 2, 3-double bonds showed a bit higher inhibitory activity compare with ampelopsin but ampelopsin glucoside showed higher inhibitory activity, 1.7 and 2.1-times, than quercetin and ampelopsin, respectively. The attached glucose at 4'-OH of B-ring enhanced NA inhibitory activity. EGC lacking the C(4)=O in the C-ring and galloy moiety group showed lowest inhibitory activity compared to that ampelopsin, quercetin, puerarin. C(4)=O in the C-ring is important to enhance inhibitory activity.

4. Conclusion

For the first time, the active extracellular NA of H5N1 influenza virus strain A/Vietnam/1203/04 was successfully expressed in *P. pastoris* X33. Among the investigated flavonoids, GCG was shown as the best comparative inhibitor against NA by *in vitro* assay. The structure and inhibition activity relationship among eight flavonoid compounds was also investigated. GCG showed numerous hydrophobic and H-bonds interaction with amino acid residues in active site pocket of NA.

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