

## ANTIOXIDANT AND ANTIVIRAL ACTIVITY OF COMMON POLYPHENOLS AGAINST INFLUENZA A VIRUS

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### SUMMARY

In this study, we compared the antioxidant activity, cytotoxicity, and antiviral activity of ten common phenolic acids and flavonoids which naturally occurred in many plants and byproducts sources. Antioxidant activity was analyzed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis (3 ethylbenzothiazoline-6-sulfonic acids) (ABTS) radical-scavenging activity, while cytotoxicity and antiviral effects of the polyphenols were evaluated using MDCK cells line model. The results showed that different phenolic acids and flavonoids showed a significant difference in antioxidant activities as well as cytotoxicity. Amongst tested compounds, pyrogallol and catechin showed higher DPPH radical scavenging ability, while ellagic acid and quercetin had the higher ability for ABTS assay. Furthermore, our results indicated that the antiviral activity potential varied significantly among polyphenols, and that several compounds including gallic acid, quercetin, rutin and vanillic acid have potent antiviral activity against the influenza A virus.

**Keywords:** *antioxidant, antiviral activity, cytotoxicity, flavonoids, influenza, phenolics.*

### 1. INTRODUCTION

Polyphenols have various clinical properties such as anti-oxidant, anti-inflammatory, antitumor, and antiviral. Due to this research on natural phytoconstituents (such as medicinal plants, native flora, and marine materials) are considered as a potential source in the development of functional foods and treatment of various diseases. Polyphenols such as catechin, gallic acid, rutin, quercetin, pyrogallol, caffeine, etc. are naturally occurring plant metabolites widely available in fruits, vegetables, nuts, and in many agro-industrial byproducts such as peels, seeds, leave, wine pomace, tea residues, and coffee spent. Polyphenols are well-known for their antioxidant properties due to the ability to scavenge free radicals. Many previous studies have shown that phytochemicals from plants and vegetables are believed to provide potential antioxidant. Also, it is known that phenolic acids, flavonoids produce a broad spectrum of unique biological effects such as antioxidant, anticancer, antimicrobial, etc. (Lin *et al.*, 2016); (Friedman, 2007). Still, much interesting is remaining to find out new sources and new methods to assess and isolate antioxidant from natural for a variety of applications. Earlier, it has been opined that the difference in the structure of phenolic components, as well as the methodology of the antioxidant assay, may cause different results in the assessment of antioxidant ability (Celep, Aydin and Yesilada, 2012).

In recent years, the utilization of phenolics and flavonoids from natural resources has become a hot issue due to its pivotal roles in many physiological, biological and pathological processes (Lin *et al.*, 2016); (Friedman, 2007). Studies have demonstrated the antioxidative activities and main mechanisms, however, exploring the benefits from nature, and especially, exploiting natural waste as agro-industrial byproducts as a potential source for therapeutic application is still a highly required in order to support further understand their metabolism and defense/safety properties in the human (Lin *et al.*, 2016).

On the other hand, influenza viruses (IVs) remain a significant threat that can cause severe morbidity and mortality responsible for epidemics and pandemics worldwide (Ju *et al.*, 2017). IVs are the group of enveloped RNA viruses belonging to the *Orthomyxoviridae* family is one of the most common infectious respiratory diseases. At present there are two main methods for control and treatment of influenza viruses include vaccination and antiviral drugs (DIng *et al.*, 2017). Studies have reported that the slow response to vaccines in dealing with epidemic outbreaks and the reducing the effectiveness of vaccination. Moreover, the resistance of IVs to current anti-IVs drugs has been emerging, and seasonal influenza viruses continue to cause epidemics around the world each year (DIng *et al.*, 2017). Therefore, it has been extensive interest in developing a new antiviral treatment for

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this virus. The biomaterials, especially from agro-industrial byproducts have been considered to be potential candidates for novel treatment against IVs infection. In this research, our goal is to evaluate the biological properties (antioxidant and antiviral activities) of ten common polyphenols, which naturally occurs in many byproducts and waste foods and has, in turn, allowed for the researchers to further investigate the specific molecules from natural compounds for the treatment of influenza virus infection.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

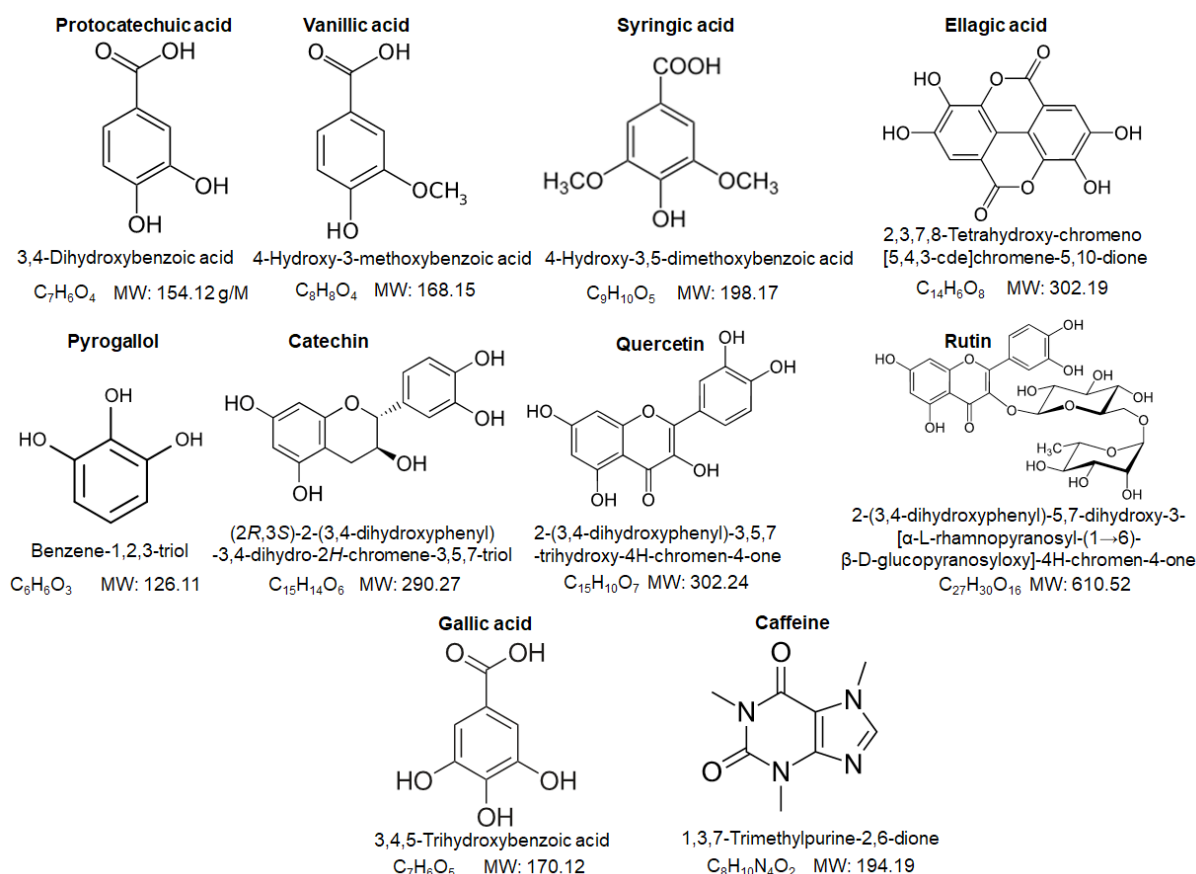


Figure 1. The chemical structures of phenolic compounds studied

### 2.2. Cells and virus

Madin–Darby canine kidney (MDCK) cells were obtained from Korean Cell Line Bank (Seoul, Korea; KCBL10034, Lot No. 30419). MDCK cells were cultured using standard methods at 37°C in 5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Korea), supplemented with 10% fetal bovine serum (FBS), and antibiotics (streptomycin 100 mg/mL and penicillin 100 U/mL, Sigma-Aldrich, St. Louis, MO, USA).

The low pathogenic human IAV H<sub>1</sub>N<sub>1</sub> strain A/PR/8/34 was purchased from American

All compounds and reagents using in this study including 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis (3 ethylbenzothiazoline-6-sulfonic acids) (ABTS), potassium persulfate, protocatechuic, vanillic acid, syringic acid, ellagic acid, pyrogallol, catechin, quercetin, rutin, gallic acid, caffeine, and oseltamivir were purchased from Sigma-Aldrich with the purity ≥98% (HPLC); All the other chemicals including solvents used were of analytical grade. Fig. 1 shows the chemical structures of ten polyphenols were used in this study.

Type Culture Collection (ATCC, Manassas, VA, USA) and propagated by infecting MDCK cells at 37 °C in 5% CO<sub>2</sub> atmosphere in virus infection media (DMEM supplemented with streptomycin 100 µg/mL, and penicillin 100 U/mL) containing trypsin treated with 2 µg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK, Sigma-Aldrich)-treated trypsin (freshly make and add TPCK right before infection), and without the supplementation of FBS.

### 2.3. DPPH radical scavenging assay

DPPH radical-scavenging ability was measured using the method of Huang, Tsai and Mau

(2006) with some modification. Serial twofold dilutions were performed to obtain the different concentration of 31.2, 62.5, 125, 250, 500, and 1000  $\mu$ M of the polyphenols. Then, one mL of each polyphenol mentioned above was mixed with one mL of freshly made DPPH solution (0.2 mM in pure methanol). The mixture was shaken and incubated in the dark for 60 min at RT. The appropriate volume of the same solvent (vehicle) used for the dilution was used instead of the samples as the blank. The absorbance then was measured at 517 nm. DPPH radical scavenging ability was calculated using the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{[\text{ABS}_{\text{ctl}} - \text{ABS}_{\text{spl}}] / \text{ABS}_{\text{ctl}}}{1} \times 100$$

Where:  $\text{ABS}_{\text{ctl}}$  is the absorbance value of the control group, and  $\text{ABS}_{\text{spl}}$  is the absorbance of the samples. The nonlinear concentration–inhibition response was plotted, and 50% inhibition concentration ( $\text{IC}_{50}$ ) was calculated.

#### 2.4. ABTS radical scavenging assay

Free radical scavenging activity of polyphenols was determined by ABTS radical cation decolorization assay using the method of Re *et al.* (1999). Briefly,  $\text{ABTS}^{\cdot+}$  cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1, v/v), stored in the dark at room temperature for 12-16 hours before use. The  $\text{ABTS}^{\cdot+}$  solution was then diluted with pure ethanol (HPLC grade) to obtain an absorbance approximately of 0.70 at 734 nm as the working solution. Different concentrations of 31.2, 62.5, 125, 250, 500, and 1000  $\mu$ M, respectively of the polyphenols were prepared. Then, 100  $\mu$ L of each compound above was mixed with one mL of ABTS working solution. The mixture was shaken and incubated in the dark for 60 min at RT. The appropriate volume of the same solvent used for the sample was used instead of the samples in the control group. The absorbance then was measured at 734 nm, and ABTS radical-scavenging ability was calculated using the following equation:

$$\text{ABTS radical-scavenging activity (\%)} = \frac{[\text{ABS}_{\text{ctl}} - \text{ABS}_{\text{spl}}] / \text{ABS}_{\text{ctl}}}{1} \times 100$$

Where:  $\text{ABS}_{\text{ctl}}$  is the absorbance value of the control group, and  $\text{ABS}_{\text{spl}}$  is the absorbance of the samples. The nonlinear concentration–inhibition response was plotted, and 50% inhibition concentration ( $\text{IC}_{50}$ ) was calculated.

#### 2.5. Cells culture and cytotoxicity assay

The cytotoxicity of polyphenols was evaluated using Madin–Darby canine kidney (MDCK) cells. Cell viability was measured by the Cell Counting

Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) method. MDCK cells were seeded in 96-wells plates at a density of  $10^5$  cells per well and incubated for 24 hours in Dulbecco's modified Eagle's medium (DMEM) with the addition of 10% heated FBS and antibiotics (streptomycin 100 mg/mL and penicillin 100 U/mL, Sigma-Aldrich, St. Louis, MO, USA). After the cell monolayer formation, cells were washed with PBS. The selected polyphenol compounds were dissolved in DMSO to 100 mM, and then serial twofold dilutions with DMEM to obtain the final concentration of 31.2, 62.5, 125, 250, 500, and 1000  $\mu$ M, respectively. The dilutions of the phenolic compounds were used to treat the MDCK cells and incubated for 48 hours at 37°C, 5%  $\text{CO}_2$ . Then 10  $\mu$ L of CCK-8 kit reagent was added to each well and after the incubation time (1 hour, at 37°C, and 5%  $\text{CO}_2$ ), the absorbance was measured at 450 nm using a microplate reader (Synergy HT, Bio-Tek, Winooski, Vermont, USA), and cytotoxicity was calculated as a percentage via the following equation:

$$\text{Cell viability (\%)} = \frac{[A-B]}{[C-B]} \times 100$$

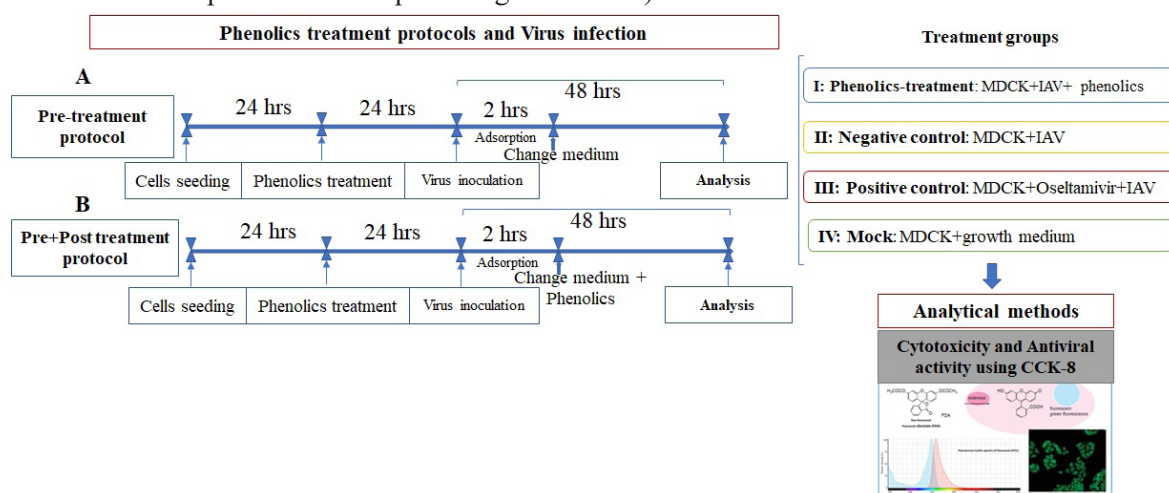
Where A, B, and C are the absorbance of the test sample (phenolic compounds treated cells), background (medium/compounds without cells), and negative control (control medium with cells), respectively. Nonlinear concentration–response curves were plotted, and half-maximal cytotoxic concentration ( $\text{CC}_{50}$ ) was calculated.

#### 2.6. Virus infection and the inhibitory effect of the selected phenolic compounds against IAV (H1N1)

For the evaluation of polyphenols against IAV infection, MDCK cells were seeded in 96-wells plates at a density of  $10^5$  cells per well and cultivated in DMEM with the addition of 10% heated FBS. After the cell monolayer formation, cells were washed with PBS. The compounds were dissolved in DMSO at a concentration of 100 mM, and the final concentration of 200  $\mu$ M was prepared in DMEM. Two treatment protocols of polyphenols (as described in the Fig. 2) were carried out: (A) pretreatment: before viral infection; (B) pretreatment + post-treatment: before and after viral infection to determine the stage at which polyphenols exerted inhibitory activity. Briefly, for the pretreatment, the medium containing ten polyphenols of the above-mentioned concentration were used to treat the MDCK cells and incubated for 24 hours at 37°C before influenza A virus infection. Then, IAV-infected MDCK cells were next incubated for 48 hours at the sample condition, and during the incubation, the virus-induced cytopathic effect (CPE) was observed using an inverted microscope (Optinity KI 400,

Korea) attached with a digital microscope camera (DCM310). After 48 hpi (hours post infection), 10  $\mu$ L of CCK-8 kit reagent was added to each well, and after the incubation time (1 hour, at 37°C, and 5% CO<sub>2</sub>), the absorbance was measured at 450 nm using a microplate reader (Synergy HT, Bio-Tek, USA) and the antiviral activity was calculated and the results were expressed as the percentage of

inhibition, while oseltamivir (C<sub>16</sub>H<sub>28</sub>N<sub>2</sub>O<sub>4</sub>·H<sub>3</sub>PO<sub>4</sub>,  $\geq 98\%$ , HPLC, Sigma-Aldrich) was used for positive control. On the other hand, for the pre+post-treatment, polyphenols were prepared and added to treat the MDCK cells at two distinct time points: 24 hours prior to virus infection (pre-treat), and at 2 hours after virus infection (post-treat).



**Figure 2. Experimental protocol and analytical methods of antiviral activity against influenza A virus of phenolic compounds**

## 2.7. Statistical analysis

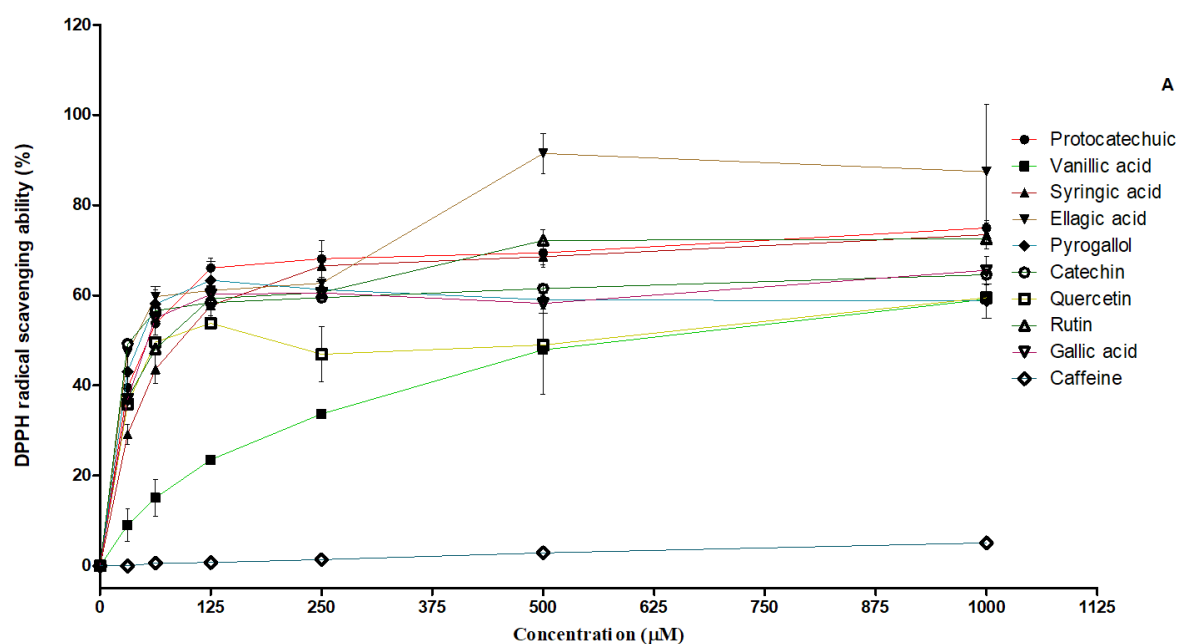
All experiments were carried out in triplicate and data were analyzed using one-way analysis of variance (ANOVA). The significant differences were assessed by the Duncan test at  $p$ -value  $< 0.05$  using Statistical Package for the Social Sciences software for Windows (SPSS IBM version 21.0). Results were presented as the mean  $\pm$  standard deviation (SD). The IC<sub>50</sub>/CC<sub>50</sub> values were calculated using Graph-Pad Prism software

version 5.01 (Graph-Pad Software Inc., USA).

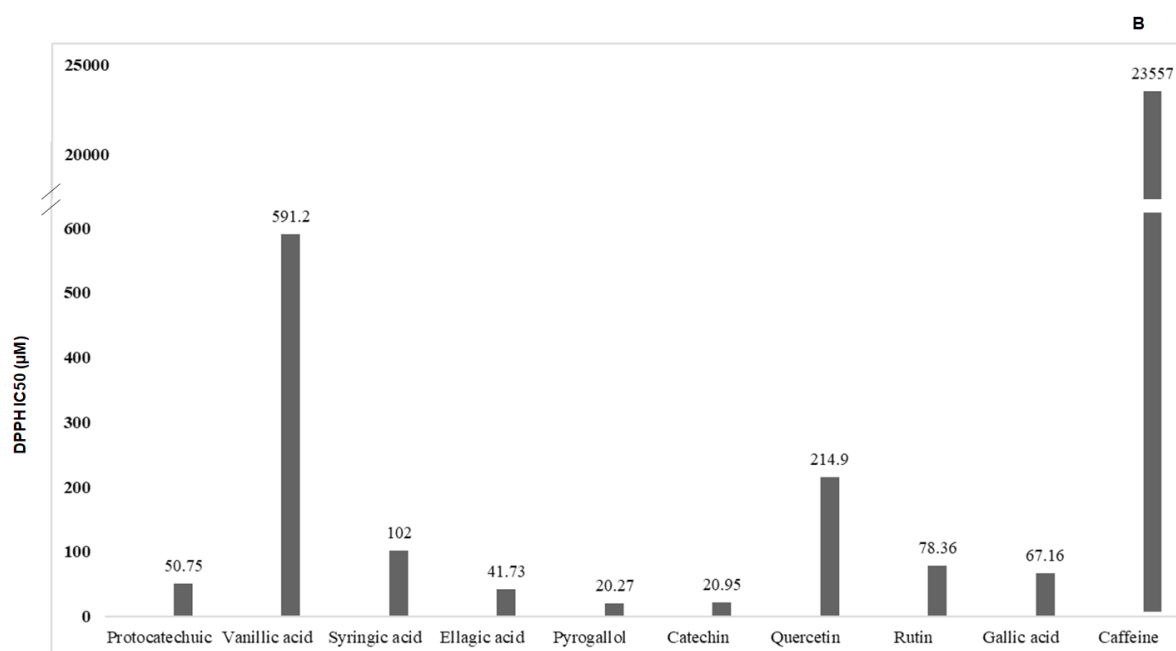
## 3. RESULTS AND DISCUSSION

### 3.1. Antioxidant activity of polyphenols

Since DPPH and ABTS radical scavenging ability are the most commonly used methods to evaluate the antioxidant activity of various sources, herein we investigated the DPPH and ABTS radical scavenging ability of polyphenols of different concentration.



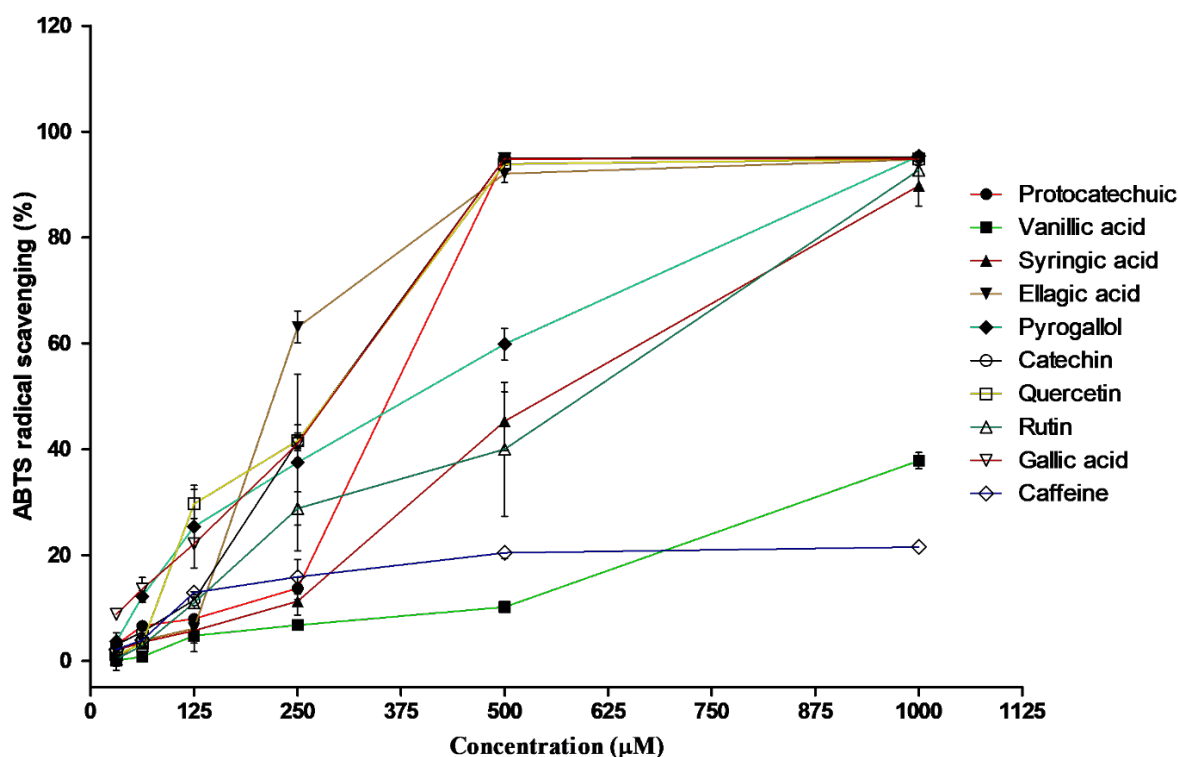


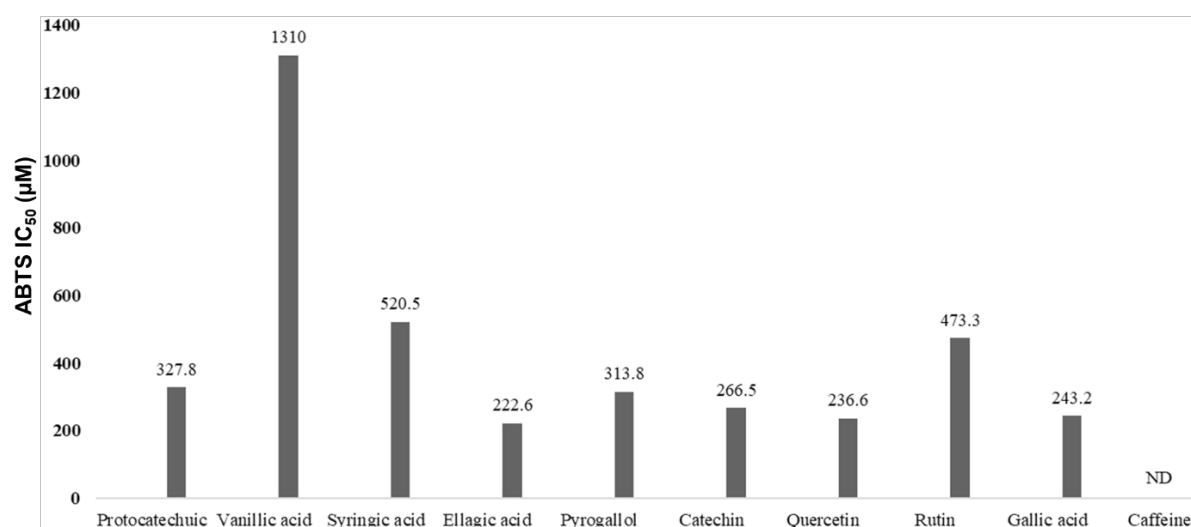


**Figure 3. DPPH radical scavenging ability. DPPH radical scavenging ability of polyphenols at different concentration (A) and together with its IC<sub>50</sub> values (B). Values (Mean ± SD of triplicate)**

The DPPH ability of ten different phenolic compounds was presented by percent inhibition (Fig. 3A) and IC<sub>50</sub> values (in the Fig. 3B). As the results are shown in the Fig 3A and B, pyrogallol and catechin were the potent antioxidant compounds, followed by ellagic acid and protocatechuic. It has been confirmed that pyrogallol and catechin are active flavonoid constituents and present high oxygen absorbing

(Friedman, 2007); (Gaikwad, Singh and Lee, 2017). In addition, a linear correlation between the concentration of the polyphenols (31.2 to 1000 μM) and discoloration of the DPPH solution was observed (Fig. 3A). This means that the radical-scavenging ability increased with increasing concentration of all polyphenols. Moreover, the results showed that different polyphenols showed a significant difference in antioxidant ability.



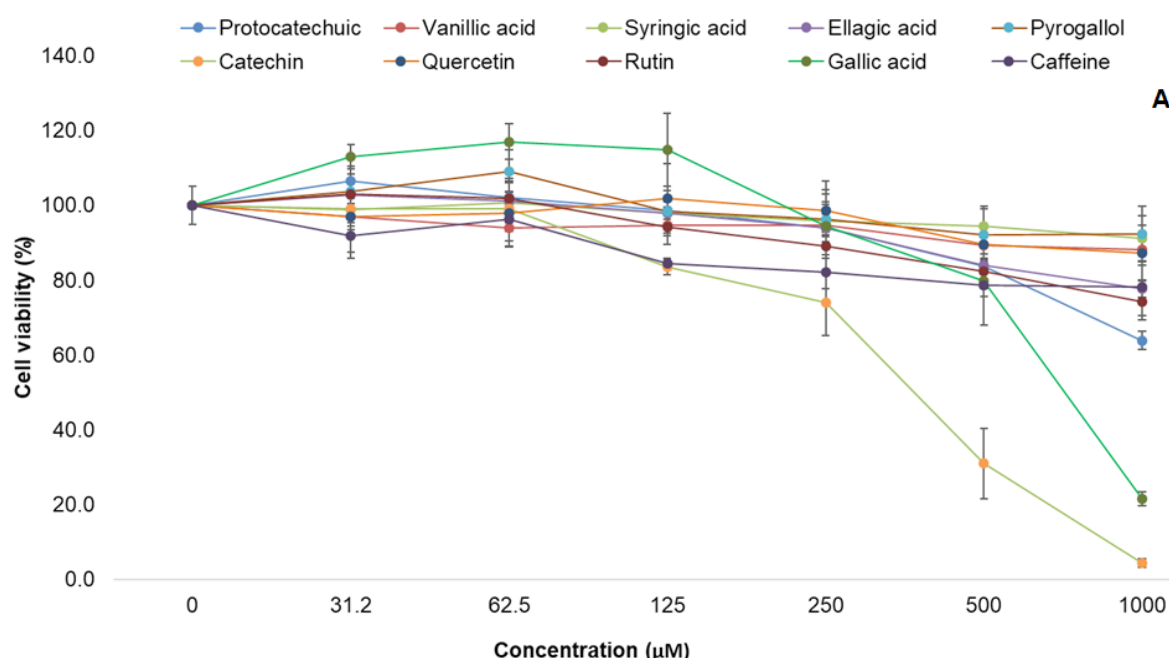


**Figure 4. ABTS radical scavenging ability. ABTS radical scavenging ability of polyphenols at different concentration (A) and together with its IC<sub>50</sub> values (B). Values (Mean ± SD of triplicate)**

As the results are shown in Fig. 4A and B, ellagic acid, quercetin and gallic acid are the most three compounds possessed the highest ABTS radical scavenging ability, which had IC<sub>50</sub> values of 222.6, 236.6, and 243.2 μM, respectively. In contrast, caffeine had no ABTS radical scavenging ability, and vanillic acid had a low ability of ABTS radical scavenging with an IC<sub>50</sub> value of 1310 μM. Moreover, consistent with results from DPPH assay, ABTS radical scavenging ability of polyphenols showed a concentration-dependent manner (Fig. 4A). Also, ABTS result was consistent with the DPPH result that confirming the different polyphenols

possessed the different antioxidant activity. The current result was in agreement with previous studies, which reported that the differences in the structure of phenolic components might have been the basis of the different results in the assessment of antioxidant ability (Celep, Aydin and Yesilada, 2012). The differences in the structure of these compounds (that have been shown in the Fig. 1), i.e., compounds contain more hydroxyl groups that have higher antioxidant activity. Due to this, the compounds structure might play an an appreciable role in the antioxidant activity.

### 3.2. Cytotoxicity and antiviral activity of polyphenols



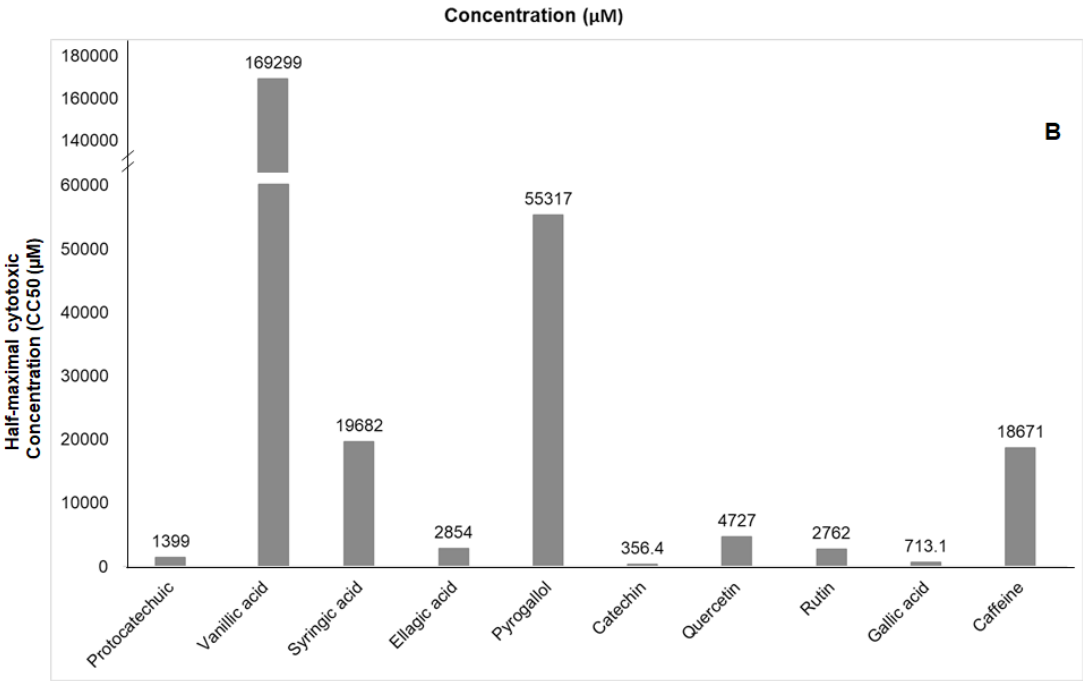


Figure 5. Cytotoxicity of phenolics and flavonoids on MDCK cells. MDCK cells were treated with different concentration of phenolics and flavonoids for 48 hours, and CCK-8 was added to measure cells viability. Cell viability was expressed as a percentage of the viability of Mock (blank control). A: Cell viability (%) of different concentration of the extracts, B: half-maximal cytotoxic concentration (CC<sub>50</sub>). Each value represents the mean ± SD of triplicate.

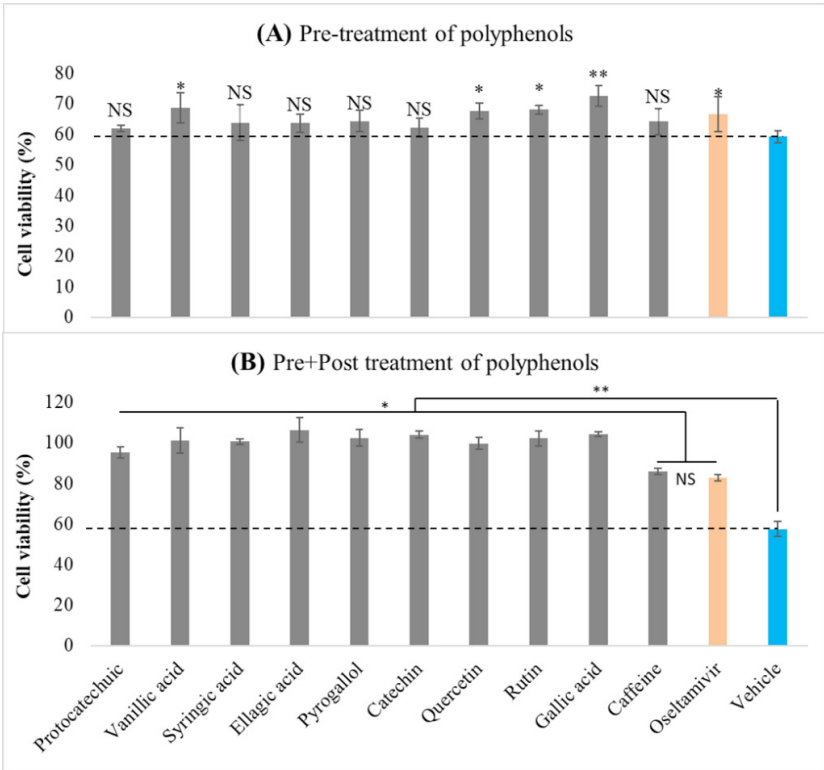


Figure 6. Inhibitory activity against influenza A virus of phenolics and flavonoids on MDCK cells. MDCK cells were treated with different phenolics and flavonoids via two treatment protocols: (A) pretreatment, (B) pre+post-treatment; then cells were infected with influenza A virus (with a multiplicity of infection of 1). The IAV-infected MDCK cells were next incubated for 48 hours at the sample condition, and CCK-8 kit was added to measure cells viability. Cell viability was expressed as a percentage of the viability of the Mock (without virus infection). Each value represents the mean ± SD of triplicate.

As shown in Fig. 5A and B, catechin was the most potent cytotoxic compound as the lowest  $CC_{50}$  value of 356.4  $\mu$ M and was toxic to the MDCK cells at the doses higher than 62.5  $\mu$ M. The cytotoxicity is followed by gallic acid with  $CC_{50} = 713.1 \mu$ M, whereas the other compounds had low or no toxic effect on MDCK cells. Interestingly, the results showed that gallic acid at low concentration (31.2 to 125  $\mu$ M) induced higher growth of MDCK cells; however, had cytotoxicity to cells at the concentration higher than 250  $\mu$ M, resulting in reduce of cell viability (Fig. 5A).

Using the CPE inhibition (the results obtained by CCK-8 assay), we tested the effect of ten compounds against the infection of influenza A ( $H_1N_1$ ) virus. As the results are shown in Fig. 6A and B, we found that gallic acid, rutin, quercetin and vanillic acid can inhibit viral penetration into the cells (as the result of pre-treatment, Fig. 6A), and gallic acid showed the highest inhibitory effect. So that, these compounds may act by interfering with the binding of virus particles to the surface of the cell. Meanwhile, other polyphenols showed no inhibitory effect against virus entry. Also, the inhibitory effect of polyphenols against the viral replication was found to highly increase when combining pretreatment and post-treatment. In fact, the cell viability of polyphenols treated groups were much higher than the control (vehicle) and even showed a significantly higher than the positive control (oseltamivir-treated) except for caffeine group (Fig. 6B). Moreover, by comparing the effect of both treatment protocols, we confirm whether the phenolic compounds were able to block virus adsorption to cells or inhibit viral replication after virus entry. As the results

clearly demonstrated that combined (pre+post) treatment (Fig. 6B) of polyphenols were more effective to inhibit the replication of influenza A virus than only the pre-treatment (Fig. 6A). Briefly, the cell viability of (pre+post) treatment had substantially higher than pretreatment. It is, therefore, polyphenols showed stronger inhibitory activity, virus replication step than virus binding step. Furthermore, the mechanism of antiviral action of natural polyphenols is based on their abilities to act as antioxidants, to inhibit enzymes, to disrupt cell membranes (as a virus receptor)/to disrupt virus particles, to neutralize and inactive the virus, to prevent viral binding and penetration into cells, and to trigger the host cell self-defense mechanisms (He *et al.*, 2011); (Lee *et al.*, 2016).

#### 4. CONCLUSION

The current study showed results of the common natural polyphenols against influenza A virus. Among constituents, gallic acid might be the most active compound since it suppressed virus entry as well as the early and later steps of the viral life cycle. This study provided that gallic acid has the anti-influenza virus activity, however the exactly inhibitory mechanisms of the compound still have not known. Thus, more specific experiments in both *in vitro* and *in vivo* of the mechanisms providing protective effects against influenza virus infection requires further investigation. On the other hands, we suggests more characterization and utilization of the active compounds from natural byproducts in the Central Highlands of Vietnam may provide effective alternatives as replacements for currently available anti-influenza A virus treatments.



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