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Chemical Constituents and Anti-influenza Viral Activity of the Leaves of Vietnamese Plant *Elaeocarpus tonkinensis*

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Abstract: Various *Elaeocarpus* species including *Elaeocarpus tonkinensis* have been important medicinal plants that used in traditional medication system and mainly used to cure nervous system-related disorders. However, their antiviral potential has not been reported yet. During the screening of medicinal plant extracts with the antiviral activity against influenza viruses, we found that *E. tonkinensis* extract has strong antiviral activity. Through organic solvent partition and repeated column chromatography using SiO_2, C-18 and Sephadex LH-20, a total of nine compounds were purified from the methanol extract of *E. tonkinensis*. Their chemical structures were determined by NMR and MS spectral data to be trolliamide (**1**), gallic acid (**2**), urolithin M-5 (**3**), hydroquinone (**4**), 2,4-dihydroxybenzoic acid (**5**), 3,5-dihydroxy-4-methoxybenzoic acid (**6**), corilagin (**7**), chebulagic acid (**8**), and shikimic acid (**9**). Their antiviral activity against influenza virus strains A/Puerto Rico/8/34 (H1N1; PR8), A/Hong Kong/8/68 (H3N2; HK) and B/Lee/40 (Lee) was examined on the basis of cytopathic effect (CPE) assay. Among them, compounds **2**, **3**, **4**, **7**, and **8** significantly inhibited viral replication in a dose-dependent manner with EC_{50} values ranging from 7.8 to 59.6 μg/mL against influenza A and/or B viruses with selectivity indices above 5.0. This study suggests that the botanical materials of *E. tonkinensis* could be promising inhibitors of influenza A and B viruses and applied to the development of a novel herbal medicine.

Keywords: Influenza virus; *Elaeocarpus tonkinensi;,* antiviral activity; infectious disease.

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1. Introduction

Influenza occurs with seasonal variations and reaches peak prevalence in winter with many people killed worldwide every year. To date, only a few organic compounds including amantadine, rimantadine, zanamivir, and oseltamivir phosphate (OSV-P) have been used for influenza therapy. However, drug-resistant influenza viruses are generated quickly. Thereby, it is attractive and urgent to seek new influenza drugs. In our screening of plant extracts against influenza viruses, we found that the methanol extract and EtOAc layer of *E. tonkinensis* exhibited antiviral activity.

Elaeocarpus tonkinensis A. DC. (*E. tonkinensis*) commonly called Com Bac Bo, is a member of family Elaeocarpaceae. It is small timber with a height of 10 m and white yellow and soft wood. In Viet Nam, it is mainly distributed in Nghe An, Thanh Hoa, Tuyen Quang, Bac Thai and Quang Ninh provinces at 100-500 m [1]. Various *Elaeocarpus* species have been used in traditional medication system such as to cure infectious diseases and nervous system related disorders. Studies indicated that *Elaeocarpus* species contain various chemical constituents including triterpenes, tannins, alkaloids, flavonoids, and glycosides [2,3], with different therapeutic activities: cytotoxic [4], anti-microbial [5], antidepressant [6], anti-diabetic [7,8], analgesic [9], asthma [10] effects. However, their anti-influenza viral activity has not been reported yet.

During the screening of plant extracts against influenza viruses, we found that the methanol extract and ethyl acetate (EtOAc) layer of *E. tonkinensis* exhibited the significant antiviral activity. Therefore, the objectives of this study were to examine the antiviral activities of its crude extracts against influenza virus strains including A/Puerto Rico/8/34 (H1N1; PR8), A/Hong Kong/8/68 (H3N2; HK) and B/Lee/40 (Lee), and to isolate and identify effective metabolites.

2. Materials and Methods

2.1. General Experimental Procedures

Silica gel 60 Å grade (particle sizes 40–63 μm) for column chromatography was purchased from Merck (Darmstadt, Germany). Sephadex LH-20 beads (size 25–100 μm) were purchased from Sigma-Aldrich (St Louis, MO). Medium Pressure Liquid Chromatography (MPLC) (CombiFlash® Rf+ system with a column C18 (43 g); 40-60 μm) system was used for isolation of active metabolites. HPLC system used for isolation and analysis consisted of a Hitachi L-2455 diode array detector, L-2130 HPLC pump, EZchrom elite data system with a ϕ 4.6 × 150 mm (TOSOH, RP-18, Japan), ϕ 10 × 150 mm (Cosmosil, RP-18, Japan) column. Thin-layer chromatography (TLC) plates (silica gel 60 F_{254}, thickness 0.2 mm) were obtained from Merck (Germany). Chemical spots on TLC plates after development were detected using *p*-anisaldehyde-sulfuric acid (AS) and ferric chloride staining reagents, or with 10% H_2SO_4 in methanol. All solvents were distilled and purified before use.

2.2. Plant Material

Leaves and twigs of *E. tonkinensis* were collected in Vu Quang National Parks (Ha Tinh), Vietnam and identified by Dr. Do Ngoc Dai, Department of Forestry, Nghe An University of Economics. A voucher specimen (No.1-MS.104.01-2014.34) has been deposited in the School of Chemical Engineering, Hanoi University of Science and Technology.

2.3. Extract Preparation and Isolation of Pure Compounds

Dried and powdered leaves and twigs of *E. tonkinensis* (9.5 kg) were extracted with MeOH−H_2O (8:2) at room temperature (3 × 24 h). The methanol extracts were combined and concentrated under vacuum at 55°C. The obtained residue was suspended in water and successively partitioned with *n*-hexane, dichloromethane (DCM) and ethyl acetate (EtOAc) to afford 247.8 g *n*-hexane, 50.1 g DCM, and 241.4 g EtOAc residues, respectively. The *n*-hexane residue (20 g) was fractionated on a silica gel column [600 g silica gel 60 Å (40–63 μm)] and eluted using mixtures of *n*-hexane−DCM (1:0 and 0:1,

v/v) and DCM−MeOH (1:0 and 9:1, v/v) to give 70 fractions (*Fr.1 – Fr.70*). Fraction *Fr.44* (2.5 g) was repeatedly chromatographed on a silica gel column [50 g silica gel 60 Å (40–63 μm)] with mixtures of *n*-hexane−DCM (1:0 and 0:1, v/v) and DCM−MeOH (1:0 and 9:1, v/v) yielding 20 mg compound **1**.

The EtOAc residue (20 g) was subjected to column chromatography (400 g silica gel 60 Å (40–63 μm) with DCM−MeOH (100:0 and 7:3, v/v), giving 40 fractions. Fraction *Fr.15* (0.6 g) was subjected to sephadex LH-20 chromatography eluting with DCM−MeOH (1:1, v/v) to give 15 fractions. The fractions *Fr.15.6* and *Fr.15.10* were combined and crystallized from DCM−MeOH (9:1, v/v) to give crystals of compounds **2** and **3**, respectively. *Fr.23* (0.4 g) was applied to a MPLC system [43 g *C-18* (40–60 μm), 1 × 15 cm, eluting with MeOH−H_2O (2:8 and 1:0, v/v)], then purified by preparative HPLC with 150×10 mm C-18 column, MeOH−H_2O stepwise gradient (1:9 to 3:7, v/v), and flow rate of 3.0 mL/min. Compounds **4** (29.1 mg), **5** (8.9 mg), and **6** (4.3 mg) were eluted at RT 21 min, RT 25 min and RT 35 min, respectively. Fraction F34 was also chromatographed by MPLC with a stepwise gradient elution of MeOH−H_2O (2:8 to 100:0, v/v) to give 3 fractions (*Fr.34.1-Fr.34.3*). *Fr.34.1* (420 mg) was subjected to HPLC to give 35.6 mg of compound **7** at RT 25 min and 10 mg of compound **8** at RT 35 min.

2.4. Structure Determination and Characterization of Isolated Compounds

The chemical structures of the isolated compounds were determined by spectroscopic methods, including mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy, and by comparison of their spectral data with values previously reported. High-resolution MS (HRMS) spectra were recorded using a Shimadzu LCMS-IT-TOF instrument (Kyoto, Japan) at atmospheric pressure chemical ionization (APCI) conditions. Ion source temperature was 300 °C, the range of detection was *m/z* 100–1200, the potential in the ion source was 4.5 kV. The drying gas (N_2) pressure was 25 kPa. The nebulizer gas (N_2) flow was 2 L/min. The ^{1}H and ^{13}C-NMR, COSY, HMQC and HMBC spectra were recorded in deuterated NMR solvents using a Bruker AMX-500 FT-NMR spectrometer (Bruker Analytische Messtechnik Gmbh, Rheinstetten, Germany).

*Trolliamide (**1**):* White powder, negative HRESIMS *m/z* 680.6267 $[M-H]^-$ ($C_{42}H_{82}NO_5$); MS-MS *m/z*: 438.3970, 426.3982, 408.3839, 383.3558, 378.3768, 365.3436, 337.3475, 333.3180, 306.2467, 265.2134, 253.2201; ^{1}H-NMR (C_5D_5N, 500 MHz), *δ* (ppm): 4.51 and 4.43 (m, H-1), 5.12 (m, H-2), 4.37 (m, H-3), 4.62 (m, H-4), 2.28 (m, H-5), 1.92 (m, H-6), 2.17 (m, H-7), 5.53 (m, H-8), 5.53 (m, H-9), 1.99 (m, H-10), 1.30-1.20 (H-11 – H-17), 0.87 (m, H-18), 4.29 (m, H-2′), 2.03 (m, H-3′), 1.75 (m, H-4′), 1.30-1.20 (H-5′ – H-23′), 0.87 (m, H-24′), 8.59 (d, *J* = 9.0, NH); ^{13}C-NMR (C_5D_5N, 125 MHz), *δ* (ppm): 61.79 (C-1), 52.57 (C-2), 76.63 (C-3), 72.80 (C-4), 33.93 (C-5), 26.29 (C-6), 33.61 (C-7), 130.59 (C-8), 130.47 (C-9), 33.08 (C-10), 30.11-29.28 (C-11 – C-15), 32.76 (C-16), 22.71 (C-17), 14.05 (C-18), 174.99 (C-1′), 72.24 (C-2′), 35.49 (C-3′), 25.59 (C4′), 30.11-29.28 (C-5′ – C-21′), 31.89 (C22′), 22.71 (C-23′), 14.05 (C-24′).

*Gallic acid (**2**):* ^{1}H-NMR (CD_3OD, 500 MHz), *δ* (ppm): 7.06 (1H, s, H-2, H-6); ^{13}C-NMR (CD_3OD, 125 MHz), *δ* (ppm): 122.1 (C-1), 110.4 (C-2, C-6), 146.30 (C-3, C-5), 139.5 (C-4), 170.6 (C-7).

Urolithin M-5 (3): ^{1}H-NMR (CD_3OD, 500 MHz), *δ* (ppm): 8.44 (1H, d, *J* = 9.0 Hz, H-1), 6.77 (1H, d, *J* = 9.0 Hz, H-2), 7.37 (1H, s, H-7); ^{13}C-NMR (CD_3OD, 125 MHz), *δ* (ppm): 119.2 (C-1), 112.5 (C-2), 144.0 (C-3), 133.3 (C-4), 140.9 (C-4a), 163.9 (C-6), 112.0 (C-6a), 108.2 (C-7), 146.4 (C-8), 146.7 (C-9), 141.9 (C-10), 118.5 (C-10a), 112.8 (C-10b).

*Hydroquinone (**4**):* ^{1}H-NMR (CD_3OD, 500 MHz), *δ* (ppm): 7.08 (1H, *s*, H-2, H-3, H-5, H-6); ^{13}C-NMR (CD_3OD, 125 MHz); *δ* (ppm): 146.4 (C-1, C-4); 110.3 (C-2, C-3, C-5, C-6).

*2,4-Dihydroxybenzoic acid (**5**):* ^{1}H-NMR (CD_3OD, 500 MHz), *δ* (ppm): 7.42 (1H, d, *J* = 2.0 Hz, H-3), 7.44 (1H, dd, *J* = 8.5; 2.0 Hz, H-5), 6.80 (1H, d, *J* = 8.5, H-6); ^{13}C-NMR (CD_3OD, 125 MHz); *δ* (ppm): 123.2 (C-1), 151.5 (C-2), 123.9 (C-3), 146.0 (C-4), 117.7 (C-5), 115.7 (C-6), 170.3 (C-7).

*3,5-Dihydroxy-4-methoxybenzoic acid (**6**):* ^{1}H-NMR (CD_3OD, 500 MHz), δ (ppm): 7.04 (2H, s, H-2, H-6), 3.82 (3H, s, 8-CH_3); ^{13}C-NMR (CD_3OD, 125 MHz); δ (ppm): 121.5 (C-1), 110.0 (C-2, C-6), 146.5 (C-3, C-5), 169.0 (C-7), 52.2 (C-8).

*Corilagin (**7**):* ^{1}H-NMR (500 MHz, CD_3OD), δ (ppm): 3,6- hexahydroxydiphenoyl (3,6-HHDP): 6.72 (H-5), 6.69 (H-5′); glucose: 6.41 (H-1), 4.00 (H-2), 4.88 (H-3), 4.50 (H-4), 4.58 (H-5), 4.21 (H-6a), 5.00 (H-6b); 1-*O*-galloyl: 7.09 (H-2‴ and H-6‴). ^{13}C-NMR (125 MHz, CD_3OD), δ (ppm): 3,6-HHDP: 115.8 (C-1), 143.9 (C-2), 136.8 (C-3), 144.2 (C-4); 108.8 (C-5), 124.1 (C-6), 167.1 (C-7), 115.3 (C-1′), 143.8 (C-2′), 136.3 (C-3′), 144.7 (C-4′), 107.0 (C-5′), 124.1 (C-6′), 168.7 (C-7′); glucose: 93.7 (C-1), 68.1 (C-2), 70.2 (C-3), 61.1 (C-4), 74.8 (C-5), 63.6 (C-6); 1-*O*-galloyl: 119.3 (C-1‴), 109.6 (C-2‴ and C-6‴), 145.0 (C-3‴ and C-5‴), 139.0 (C-4‴), 165.4 (C-7‴).

*Chebulagic acid (**8**):* ^{1}H-NMR (500 MHz, CD_3OD), δ (ppm): 3,6-HHDP: 6.84 (H-5), 6.64 (H-5′); glucose: 6.51 (H-1), 5.39 (H-2), 5.83 (H-3), 5.23 (H-4), 4.90 (H-6a), 4.39 (H-6b); 1-*O*-galloyl: 7.08 (H-2‴ and H-6‴), 2-*O*-4-*O*-chebuloyl: 5.06 (H-3′), 3.81 (H-4′), 2.00 (H-5a′), 1.20 (H-5b′), 7.48 (H-2″). ^{13}C-NMR (125 MHz, CD_3OD), δ (ppm): 3,6-HHDP: 117.6 (C-1), 145.5 (C-2), 138.6 (C-3), 145.6 (C-4), 110.4 (C-5), 124.5 (C-6), 167.5 (C-7), 116.2 (C-1′), 145.3 (C-2′), 137.5 (C-3′), 146.1 (C-4′), 108.2 (C-5′), 125.6 (C-6′), 170.1 (C7′); glucose: 92.5 (C-1), 71.1 (C-2), 62.4 (C-3), 66.8 (C-4), 74.5 (C-5), 64.7 (C-6); 1-*O*-galloyl: 120.1 (C-1‴), 110.9 (C-2‴and C-6‴), 146.5 (C-3‴and C-5‴), 140.8 (C-4‴), 166.2 (C-7‴), 2-*O*-4-*O*-chebuloyl: 170.7 (C-1′), 67.0 (C-2′), 41.7 (C-3′), 40.0 (C-4′), 30.7 (C-5′), 174.9 (C-6′), 174.4 (C-7′), 119.0 (C-1″), 117.6 (C-2″), 147.4 (C-3″), 140.4 (C-4″), 141.4 (C-5″), 115.9 (C-6″), 166.4 (C-7″).

*Shikimic acid (**9**):* ^{1}H-NMR (500MHz, CD_3OD), δ (ppm): 6.67 (1H, m, H-2), 4.36 (1H, brt, J = 4.5 Hz, H-3), 3.98 (m, H-5), 3.64 (1H, dd, J = 8.5, 4.5 Hz, H-4), 2.80 (1H, dd, J = 18.0, 5.0 Hz, H-6α), 2.23 (1H, dd J = 18.0, 7.0 Hz, H-6β); ^{13}C-NMR (500MHz, CD_3OD) δ (ppm): 134.26 (C-1), 135.22 (C-2), 67.57 (C-3), 73.54 (C-4), 68.37 (C-5), 33.12 (C-6), 172.79 (C-7).

2.5. Cells, Viruses and Antiviral Agents

Influenza viruses PR8, HK and Lee were purchased from the American Type Culture Collection (ATCC). Influenza A viral strains (PR8 and HK) were amplified by infection of 10-day-old chicken eggs at 37°C for 3 days and influenza B virus (Lee) by infection of Madin-Darby canine kidney (MDCK) cells under serum-free conditions. MDCK cells were purchased from ATCC and maintained in minimum essential medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37°C in 5% CO_2. Antiviral agents, amantadine hydrochloride (AMT), ribavirin (RBV) (Sigma-Aldrich) and oseltamivir carboxylate (OSV-C; US Biological) were applied as positive control compounds.

2.6. Cytopathic Effect (CPE) Inhibition Assay

CPE was performed according to our previous report [26]. MDCK cells were seeded in 96-well plates and were mock-infected or infected with influenza virus at a multiplicity of infection (MOI) of 0.001 for 1 h at 35°C under serum-free conditions. Test materials dissolved in MEM with 2 μg/mL TPCK-trypsin were added to each well. Fluorescence intensity reflecting cell viability was measured using fluorescein diacetate (FDA; Sigma) at 485/538 nm. The half maximal cytotoxic concentration (CC_{50}) and the half maximal effective concentration (EC_{50}) were calculated using GraphPad Prism 6 (GraphPad Software). The selectivity index (S.I.) is expressed as the ratio of CC_{50} to EC_{50}.

2.7. Statistical Analysis

Statistically significant differences were determined using two tailed Student's t-test. $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Isolation and Identification of Metabolites from E. tonkinensis

Nine pure metabolites were isolated and identified from the n-hexane and EtOAc residues of *E. tonkinensis* (Figure 1). Compound **1** was obtained as a white powder. The structure of this compound (Figure 1) can be established by 1D and 2D NMR spectra in combination with the MS data. Its molecular formula was determined as $C_{42}H_{83}NO_5$ by negative HRESIMS at *m/z* 680.6279 $[M-H]^-$. The NMR spectra suggested a ceramide structure. Thus, in the ^{1}H-NMR spectrum, the strong $(CH_2)_n$ signals at δ 1.30 and two terminal methyl signals at δ 0.87 also confirmed the ceramidic nature of this compound. The ^{1}H-NMR signal at *δ* 5.12 (1H, *m*) was assignable to H(2), which coupled with signals at *δ* 4.51 (1H, *m*, H(1)), 4.43 (1H, *m*, H(1)) and 4.37 (1H, *m*, H(3)). In the ^{13}C NMR spectrum, the signals of three characteristic ceramide groups appeared at *δ* 175.0 (amide carbon), 52.6 (nitrogenated carbon), and 29.9 (the $(CH_2)_n$ signals). C(1) and C(3) were assigned to the signals at *δ* 61.8 and 76.6, indicating C(3) of chain A was hydroxylated. The coupling of H(3) and H(4) in COSY spectrum indicated the presence of hydroxyl group at C(4). The C(2′) (*δ* 72.24) of chain B was also demonstrated to be hydroxylated from the correlation between the carbonyl signal at *δ* 175.0 (C(1)) and 4.29 (1H, *m*, H(2′)). The geometry of double bond at Δ^8 was determined to be *trans* as evidenced by allylic methylenes at *δ* 33.6 (C(7)) and 33.1 (C(10)) [11]. The structures of chain A and B were determined by the analysis of fragmentation patterns in the HRESI MS/MS spectrum (Figure 2). In this spectrum, the chain A can be seen through fragments with *m/z* 223.2080 ($C_{15}H_{17}O^-$), 253.2140 ($C_{16}H_{18}O_2^-$) and 426.3968 ($C_{26}H_{52}NO_3^-$). The chain B can be proved on the basis of fragments with *m/z* 337.350 ($C_{23}H_{45}O^-$), 365.346 ($C_{24}H_{45}O_2^-$), and 383.3578 ($C_{24}H_{47}O_3^-$). Based on the analysis of spectral data combined with literature data [12], the compound **1** was confirmed to be trolliamide.

Compounds **2**, **3**, and **6** were determined from their 1D–NMR spectra as gallic acid and its derivatives with six aromatic carbon signals and a carbonyl group. Compound **2** was identical to gallic acid on the basis of 1D–NMR data analysis and comparison with those in the literature [13]. Compound **6** was identified as methoxy derivative of compound **2**. The ^{13}C-NMR spectra of compound **3** presented of 13 carbon signals in the range δ 108.2-163.9 suggested the presence of two aromatic rings and one lactone ring. One of the aromatic rings had characteristic carbon signals of gallic acid, comprising a carbonyl group at δ 163.9 and six aromatic carbons from δ 112.0 to 146.7. The chemical structure of compound **3** was determined by comparison with literature NMR data [14,15]. Compound **3** was identical to urolithin M-5, and was obtained as light brown crystals. Compounds **4** and **5** were determined by the analysis of spectra data combined with literature data [16,17]. Compounds **4** and **5** were determined to be hydroquinone ($C_6H_6O_2$) and 2,4-dihydroxybenzoic acid ($C_7H_6O_4$), respectively.

Compounds **7** and **8** were the first time isolated from this species. Their chemical structures were described as shown in Figure 1. The NMR spectra of compound **7** showed a hexahydroxydiphenoyl-D-glucose (HHDP-D-glucose) group and a galloyl group. The ^{1}H-NMR spectra showed three singlet peaks, including two proton signals of the HHDP group at *δ* 6.69 and 6.72 and one proton signal of the galloyl group at *δ* 7.09. In addition, six signals appeared in the range of *δ* 4.00-6.41 belong to a glucose ring. The ^{13}C-NMR spectra also showed six carbon signals of the glucose ring at *δ* 61.1-93.7. Moreover, the carbon signals of the galloyl group appeared at *δ* 109.6-165.4. The fourteen remaining carbon signals were assigned to the HHDP group. The proton-bearing carbons assigned by the HMQC spectra showed the linking position of the galloyl and HHDP groups with glucose ring. A methine proton of glucose ring at *δ* 4.88 (H(3)) indicated HMBC correlations with carbon signal of the carbonyl group at *δ* 167.2 (C(A-7)) and methylene protons of glucose ring at *δ* 4.21 and 5.00 (H(6)) indicated correlations with ^{13}C-NMR signal at *δ* 168.7 (C(B-7)) led to linking position of the HHDP group with H(3) and H(6) of the glucose ring. Similarly, the galloyl group (*δ* 165.4) linked with H(1) (*δ* 6.41) of the glucose ring. From these data and the comparison of spectral data of compound **7** with literature data [18], compound **7** was determined to be corilagin. The NMR spectra of compound **8** were similar to those of compound **7** with some differences with the presence of 2-*O*-4-*O*-chebuloyl group. This analysis and comparison with literature [19] suggested compound **8** to have the same rearranged tannin structure, and compound **8** was identified as chebulagic acid.

Compound **9** was demonstrated as shikimic acid by the analysis of spectral data and comparison with literature data [20]. Its ^{1}H-NMR spectra showed a methine proton at δ 6.67, three oxygenated methin protons at δ 4.36 (*brt, J* = 4.5 Hz, H(3)), 3.98 (*m*, H(5)), 3.64 (*dd, J* = 8.5, 4.5 Hz, H(4)) and two methylene protons at δ 2.80 (*dd, J* = 18.0, 5.0, Hz, H(6α)), 2.23 (*dd, J* = 18.0, 7.0 Hz, H(6β)). The ^{13}C-NMR spectrum of **9** had seven carbon signals. At low field, a carbon signal shown at δ 172.8 belongs to carbonyl carbon. In addition, two olefin carbons also appeared at δ 134.3 and 135.2 and one methylene carbon shown at δ 33.1. The remaining carbon signals appeared at 67.6-73.5 assigned to three oxygenated carbons.

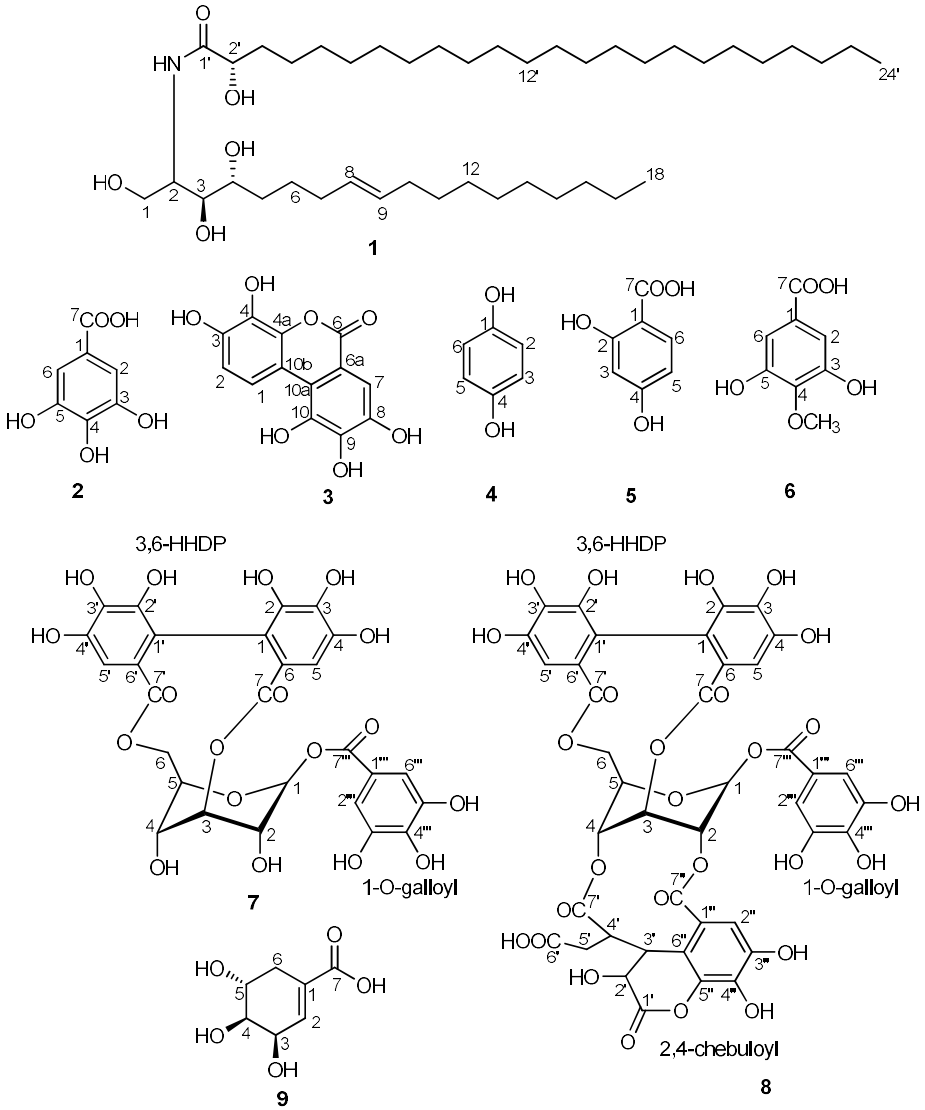

Figure 1. Chemical structures of the compounds isolated from *E. tonkinensis.*

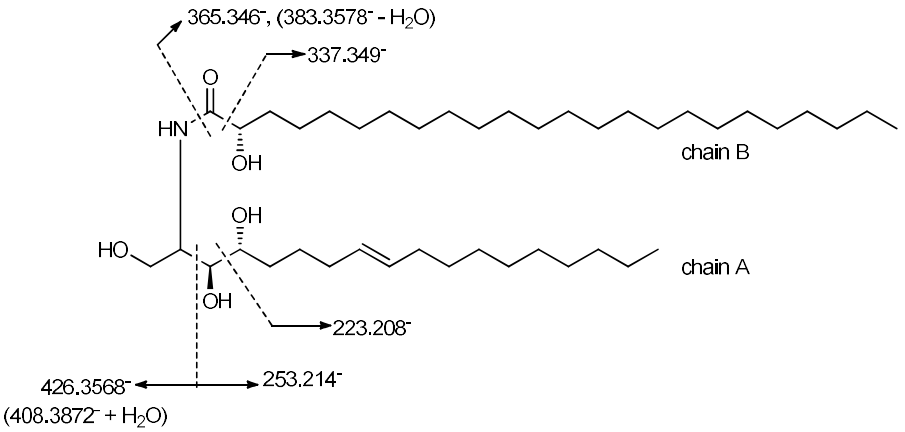

Figure 2. Selective fragmentation patterns in the mass spectra of compound **1** (trolliamide) isolated from *E. tonkinensis*. MS spectra were recorded using a Shimadzu LCMS-IT-TOF instrument at atmospheric pressure chemical ionization (APCI) conditions.

3.2. Anti-influenza Activity of Crude Extract and Residues of E. tonkinensis

Anti-influenza viral activity of *E. tonkinensis n*-hexane, DCM and EtOAc residues and MeOH extract was presented in Table 1. *n*-Hexane and DCM residues did not show any antiviral activity under the subtoxic concentrations lower than 280.0 μg/mL and 111.2 μg/mL, respectively. Among them, MeOH extract showed the most potent inhibitory effects with EC_{50} values 19.8 μg/mL for PR8, 5.1 μg/mL for HK, and 107.7 μg/mL for Lee strains. In contrast, the EtOAc residue exhibited marginal antiviral activity with EC_{50} values ranging between 52.7 μg/mL and 205.0 μg/mL for all strains. Moreover, it is noteworthy that the MeOH extract and EtOAc residue were not cytotoxic to MDCK cells at the highest concentration used (300.0 μg/mL).

Table 1. Antiviral activity of the crude extracts from *E. tonkinensis* against influenza viruses in the cytopathic effect (CPE) reduction assay.

Sample	CC_{50}[a]	EC_{50}[b] (S.I.[c])			Unit
		PR8[d]	HK[e]	Lee[f]	
Hex[g]	280.0 ± 19.3	>280.0 (N.A.[n])	>280.0 (N.A.[n])	>280.0 (N.A.[n])	μg/mL
DCM[h]	111.2 ± 6.6	>111.2 (N.A.[n])	>111.2 (N.A.[n])	>111.2 (N.A.[n])	μg/mL
MeOH[i]	>300.0	19.8 ± 1.9 (>15.2)	5.1 ± 0.3 (>59.4)	107.7 ± 40.6 (>2.8)	μg/mL
EtOAc[i]	>300.0	79.6 ± 18.3 (>3.8)	52.7 ± 3.4 (>5.7)	205.0 ± 24.1 (>1.5)	μg/mL
AMT[k]	>100.0	>100.0 (N.A.[n])	3.2 ± 1.6 (>31.3)	>100.0 (N.A.[n])	μM
RBV[l]	>100.0	55.6 ± 1.3 (>1.8)	56.9 ± 0.8 (>1.8)	52.2 ± 8.3 (>1.9)	μM
OSV-C[m]	>100.0	0.29 ± 0.05 (>344.8)	0.01 ± 0.00 (>11,764.7)	2.36 ± 1.35 (>42.5)	μM

[a]CC_{50}, 50% cell toxicity concentration; [b]EC_{50}, 50% effective concentration; [c]S.I., Selectivity index = CC_{50}/EC_{50}; [d]PR8, A/Puerto Rico/8/34 (H1N1); [e]HK, A/Hong Kong/8/68 (H3N2); [f]Lee, B/Lee/40; [g]Hex, *n*-hexane extract; [h]DCM, dichloromethane extract; [i]MeOH, methanol extract; [j]EtOAc, ethyl acetate layer derived from the methanol extract; [k]AMT, amantadine hydrochloride; [l]RBV, ribavirin; [m]OSV-C, oseltamivir carboxylate; [n]N.A., not applicable.

3.3. Anti-influenza Viral Activity of Isolated Compounds

CPE inhibition assay was further performed for evaluating anti-influenza viral activity of the 9 isolated compounds (Table 2 and Figure 3). Compounds **1** and **5** displayed marginal or no anti-influenza activity and no cytotoxicity to MDCK cells at 300 μg/mL.

Compounds **2**, **3** and **6** are belonging to gallic acid and its derivatives (Figure 1). Out of those, compounds **2** and **3**, but not **6** (displayed marginal), showed activity against influenza A and B strains with SI values above 5 against all viruses tested (Table 2). In terms of selectivity, compound **2** (gallic

acid) exhibited effectively activity against influenza with EC_{50} values of 8.1, 7.8 and 19.4 μg/mL against PR8, HK and Lee, respectively, with a CC_{50} value of about 161.4 μg/mL, that is consistent to previous reports [21,22]. Although compound **3** (urolithin M-5) showed weaker antiviral activity than compound **2**, it was remarkably active against the influenza A and B viruses with EC_{50} values of 34.3 to 59.6 μg/mL comparably. Most of all, this is the first observation supporting that urolithin M-5 has anti-influenza viral function. The anti-influenza viral activity of gallic acid and its derivatives could be associated with their planar structure and cell membrane permeability, as described previously (Kaihatsu et al. 2014). When compared to compounds **2** and **3**, another gallic acid derivative, compound **6** lost antiviral activity dramatically (EC_{50} values between 127.9 to 206.1 μg/mL), suggesting that hydroxyl group at C4 of gallic acid is essential for suppressing viral growth.

Compound **4** showed anti-influenza viral activity with an EC_{50} of 31.9, 19.7 and 54.3 μg/mL for PR8, HK and Lee, respectively. The tannin compounds, compounds **7** and **8** also showed desirable antiviral activity against PR8, HK and Lee without cytotoxicity or an abnormally increase in cell viability at the maximum concentration treated (300.0 μg/mL) (Table 2 and Figure 3). Compound **7** showed activity against influenza A virus with an EC_{50} of 31.2 and 36.8 μg/mL for PR8 and HK, respectively. Particularly, compound **8** revealed anti-influenza viral activity with EC_{50} values of 21.0, 29.0 and 58.8 μg/mL for PR8, HK and Lee, respectively. Although compound **9** showed antiviral activity against three influenza virus strains with EC_{50} values between 26.3 and 71.4 μg/mL, its cytotoxicity was severe with CC_{50} of 141.2 μg/mL as observed in compound **2**. In spite of its cytotoxicity, the anti-influenza viral activity of compound **9** was reported in previous studies [23,24]. It was also used as a primary structure to synthesize oseltamivir phosphate, an antiviral drugs with a brand name of Tamiflu [25] (Table 2 and Figure 3).

Table 2. Antiviral activity of the substances isolated from *E. tonkinensis* against influenza viruses in the cytopathic effect (CPE) reduction assay.

Chemical	CC_{50}[a]	EC_{50}[b] (S.I.[c])			Unit
		PR8[d]	HK[e]	Lee[f]	
1[g]	>300.0	>300.0 (N.A.[s])	122.1±22.1 (>2.5)	>300.0 (N.A.[s])	μg/mL
2[h]	161.4 ± 13.8	8.1 ± 0.0 (20.0)	7.8 ± 0.3 (20.8)	19.4 ± 2.2 (8.3)	μg/mL
3[i]	>300.0	58.9 ± 0.3 (>5.1)	34.3 ± 13.5 (>8.8)	59.6 ± 0.8 (>5.0)	μg/mL
4[j]	>300.0	31.9 ± 10.8 (>9.4)	19.7 ± 3.4 (>15.3)	54.3 ± 1.6 (>5.5)	μg/mL
5[k]	>300.0	>300.0 (N.A.[s])	>300.0 (N.A.[s])	154.1 ± 26.4 (>1.9)	μg/mL
6[l]	>300.0	130.2 ± 5.6 (>2.3)	127.9 ± 4.5 (>2.3)	206.1 ± 48.3 (>1.5)	μg/mL
7[m]	>300.0	31.2 ± 0.7 (>9.6)	36.8 ± 8.1 (>8.2)	160.3 ± 7.2 (>1.9)	μg/mL
8[n]	>300.0	21.0 ± 1.8 (>14.3)	29.0 ± 13.6 (>10.3)	58.8 ± 2.6 (>5.1)	μg/mL
9[o]	141.2 ± 11.5	61.4 ± 15.8 (>2.3)	26.3 ± 3.8 (>5.4)	71.4 ± 9.0 (>2.0)	μg/mL
AMT[p]	>100.0	>100.0 (N.A.[s])	2.0 ± 0.2 (>50.0)	>100.0 (N.A.[s])	μM
RBV[q]	>100.0	35.2 ± 7.9 (>2.8)	17.0 ± 0.2 (>5.9)	19.4 ± 0.5 (>5.2)	μM
OSV-C[r]	>100.0	0.21 ± 0.06 (>476.2)	<0.005 (>20,000.0)	1.08 ± 0.22 (>92.6)	μM

[a]CC_{50}, 50% cell toxicity concentration; [b]EC_{50}, 50% effective concentration; [c]S.I., selectivity index = CC_{50}/EC_{50}; [d]PR8 A/Puerto Rico/8/34 (H1N1); [e]HK A/Hong Kong/8/68 (H3N2); [f]Lee B/Lee/40; [g]**1** trolliamide; [h]**2** gallic acid; [i]**3** urolithin M-5; [j]**4** hydroquinone; [k]**5** 2,4-dihydroxybenzoic acid; [l]**6** 3,5-dihydroxy-4-methoxybenzoic acid; [m]**7** corilagin and; [n]**8** chebulagic acid; [o]**9** shikimic acid; [p]AMT amantadine hydrochloride; [q]RBV, ribavirin; [r]OSV-C, oseltamivir carboxylate; [s]N.A. not applicable.

4. Conclusions

This is the first report on isolation, identification and anti-influenza virus activity of the compounds isolated from *E. tonkinensis*. The MeOH extract and EtOAc layer of *E. tonkinensis* exhibited antiviral activity against influenza viruses of A/H1N1, A/H3N2 and B. Out of constituents isolated from the EtOAc layer, gallic acid, urolithin M-5, hydroquinone, corilagin, chebulagic acid and shikimic acid showed significant anti-influenza viral activity. Our study suggests that the botanical materials of *E. tonkinensis* could be promising inhibitors of influenza A and B viruses and applied to the development of a novel herbal medicine.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/RNP

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