Original article

# Catechol Group of Cinnamic Acid Derivative Is Essential for Its Anti-Hepatitis C Virus Activity

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**Abstract:** We previously reported that several cinnamic acid derivatives exhibited antiviral effects on hepatitis C virus (HCV). However, further analysis on the chemical structural features of these compounds are required for development of an effective anti-HCV agent. In this study, we examined the relationship between anti-HCV activity and chemical structure using 14 cinnamic acid derivatives to identify an effective anti-HCV compound. Cinnamic acid derivatives with catechol rings showed higher antiviral activities than other compounds. In addition, pyrocatechol, 1,2-dihydroxybenzene, showed antiviral activity. The most effective derivative among the compounds used in this study and our previous report synergistically inhibited HCV replication in combination with daclatasvir or interferon-$\alpha$2b, but not with telaprevir. These findings suggest that the catechol ring of cinnamic acid derivatives is critical for anti-HCV activity and that the cinnamic acid derivative synergistically inhibits HCV replication in combination with a clinically-used direct-acting antiviral agent for HCV.

**Key Words:** Hepatitis C virus, cinnamic acid, antiviral

## INTRODUCTION

Hepatitis C virus (HCV) infects at least 71 million people worldwide, leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma[1]. Current therapy for hepatitis C using effective direct-acting antivirals (DAA) can achieve sustained viral response (SVR) in over 95% of cases without any serious side effects[2]. However, several issues still remain to be solved[3].

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1) DAA therapy of patients infected with the viral genotype 3, which is a major genotype in South Asia and Europe, is limited. 2) Therapy using DAA with ribavirin improves the achievement rate of SVR, although ribavirin causes moderate side effects and is a contraindication for elderly patients and patients with renal failure. 3) Various drugs including antiepileptic drugs are contraindicated when use in combination with some DAAs. Thus, development of more efficient and safe anti-HCV agents with fewer side effects is still needed.

Cinnamic acid derivatives are reported to have multiple biological activities such as antimicrobial, anti-cancer, anti-oxidant, anti-fungal,

and anti-diabetic activities [4–8]. We recently reported that the derivatives inhibit HCV replication via the induction of oxidative stress [9].

In this study, we tested the effects of other cinnamic acid derivatives on anti-HCV activity and synergic effects of an effective derivative with currently available anti-HCV agents to identify an effective antiviral agent and to develop an effective therapy. The cinnamic acid derivatives used in this study are different from the derivatives used in our previous report [9] in combination of residues (OH, CH3, CN and others) at R1, R2, R3, and R4 (Table I) in order to clarify the effect of catechol-ring structure on antiviral effect.

## MATERIALS AND METHODS

### Cell culture and luciferase reporter assay

The Huh7/Rep-Feo cell line, which harbors the subgenomic replicon RNA of the N strain (genotype 1b), was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum, 1% of penicillin-Streptomycin solution (Sigma-Aldrich, St. Louis, USA), and 0.5 mg/mL of G418 (Nakarai, Tokyo) [9]. These cells were seeded at a density of 2.0 x $10^4$ cells per well on 48-well plates, were treated with various concentrations of compounds, and then harvested at 72 h post-treatment. Luciferase assay was carried out as described previously [9]. The cytotoxicity was evaluated by the

Table I. List of compounds used for screening
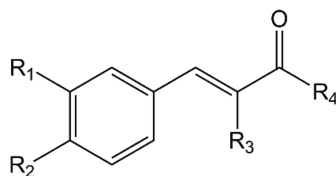


| Compound | EC50 ($\mu$M) | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|----------|---------------|-------|-------|-------|-------|
| A | 37.0 ± 0.4 | OH | OH | CN | $OC_2H_4Th$ |
| B | 33.7 ± 1.2 | OH | OH | CN | $NH_2$ |
| C | 14.5 ± 0.7 | OH | OH | CN | NHPh |
| D | 7.5 ± 0.5 | OH | OH | CN | $NHCH_2PhCF_3$ |
| E | 9.5 ± 0.2 | OH | OH | CN | $NHC_3H_6Ph$ |
| F | 6.5 ± 0.2 | OH | OH | CN | $NHC_4H_8Ph$ |
| G | 14.3 ± 0.6 | OH | OH | CN | $C_7N_3H_9Ph(OH)_2$ |
| H | 21.1 ± 0.3 | OH | OH | CN | $Ph(OH)_2$ |
| I | 8.7 ± 1.0 | OH | OH | H | $NHC_3H_6Ph$ |
| J | 73.8 ± 2.4 | H | OH | CN | $NHCH(CH_3)_2$ |
| K | 78.6 ± 3.8 | H | OH | CN | $NHC_3H_7$ |
| L | 67.5 ± 2.6 | H | OH | CN | $NHC_4H_9$ |
| M | 59.7 ± 1.0 | H | OH | CN | $NHC_2H_4Ph$ |
| N | 51.3 ± 1.0 | H | OH | CN | $NHC_3H_6Ph$ |

method described previously[9].

*HCV infection and Quantification of viral RNA*

The Huh7OK1 cell line is highly permissive for cell-cultured HCV (HCVcc) as described previously[10]. The viral RNA *in vitro* transcribed from the plasmid pJFH1 was transfected into Huh7OK1 cells for HCVcc preparation according to the method of Wakita *et al.*[11]. Huh7OK1 cells were infected with HCVcc at a multiplicity of infection of 0.1 and then passaged once every 4 days. These infected cells cultured 8 days post-transfection were employed as the HCVcc-infected cells, which exhibited more than 90% NS5A-positive cells[11]. HCVcc-infected cells were seeded at a density of $4.0 \times 10^5$ cells per well on 6-well plates and treated with various concentrations of compound 6 as reported previously[9]. Viral RNA was isolated from supernatants after 24 h treatment using the QIAamp mini viral RNA extraction Kit (Qiagen, Hilden, Germany) and reverse-transfected into cDNA using the Thermoscript reverse transcriptase kit (Thermo Fisher Scientific, Waltham, USA). The copy numbers of viral genomes were quantified using the KOD SYBR qPCR Mix Kit (TOYOBO, Osaka) and Step One Plus Real-Time PCR System (Thermo Fisher Scientific). HCV RNA and GAPDH mRNA were estimated by quantitative real-time polymerase chain reaction (qRT-PCR) utilizing the primer pairs 5'-GAGTGTCGTACAGCCTC-CA -3' and 5'-CACTCGCAAGCGCCCTATCA-3' and 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC -3', respectively.

*Combination studies*

The combined effect of compound 6 with telaprevir, daclatasvir, or IFN-$\alpha$2b was determined using the ED43/SG-Feo (VYG) cell line, which was kindly provided by C. M. Rice[12,13]. The cells were seeded at a density of 2.0 x $10^4$ cells per well on 48-well plates and treated with each drug alone, or combinations of compound 6 with telaprevir, daclatasvir, or IFN-$\alpha$2b. The treated cells were harvested 72 h after treatment. The luciferase was expressed dependently of HCV replication because the luciferase gene was polycistronically encoded on the replicon RNA together with the HCV subgenomic genome and was translated via HCV IRES. HCV nonstructural proteins were expressed by EMCV IRES located between the luciferase gene and the HCV nonstructural ORF. The luciferase activity was estimated in triplicate. The data were represented from three independent experiments.

*Reagents*

Compounds A, B, C, and F were purchased from TOCRIS Bioscience (Bristol, UK). Compounds G and H were from Sigma-Aldrich (St. Louis, MO, USA). Compound D was from Merck Millipore (Billerica, MA, USA). Cinnamic acid amide compound I was prepared by amidation between caffeic acid[14] and phenylpropylamine. 2- Cyanocinnamic acid amides compounds J, K[15], L[16], M[17], and N[18] were synthesized by Knoevenagel condensation of corresponding cyanoacetamides with *p*-hydroxybenzaldehyde. Cyanoacetic acid, p-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, phenylethylamine, phenylpropylamine, isopropylamine, propylamine, and butylamine were purchased from Wako Pure Chemical (Tokyo). Compound J was also commercially available from Aurora Building Blocks (San Diego, CA, USA). Spectroscopic data of the above known amides prepared were identical to those reported. Interferon $\alpha$-2b (IFN-$\alpha$2b) was obtained from MSD (Tokyo).

*Data analysis*
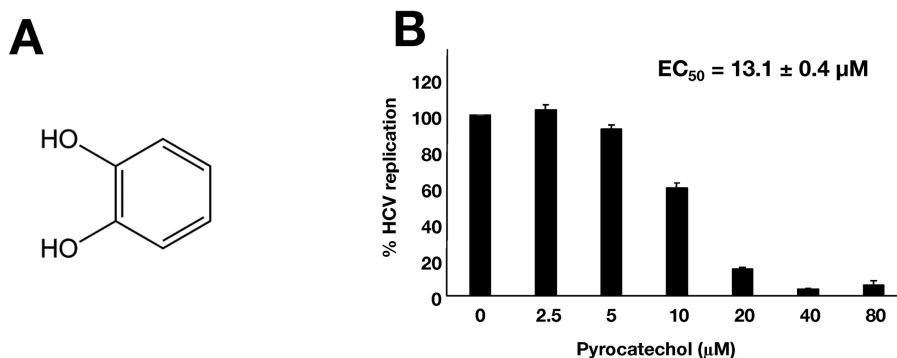
Each estimated value represented the mean

Fig. 1.  The structure and antiviral activity of pyrocatechol
(A) The structure of pyrocatechol. (B) The antiviral activity of pyrocatechol. The replicon cells were treated with an indicated concentration of pyrocatechol as described in Materials and Methods. The luciferase activity and cytotoxicity were evaluated. The $CC_{50}$ value of pyrocatechol was $89.2 \pm 1.7$ $\mu$M.

± standard deviation (SD). The statistical analysis was performed using Student's t-test. A value of P < 0.05 was defined as significant. Data of combination studies were analyzed for synergistic effects utilizing the computer software CalcuSyn software (HULINKS, Tokyo). A combination index (CI) of 0.9~1.1, CI < 0.9, and CI > 1.1 indicate an additive effect, a synergistic effect, and an antagonistic effect, respectively.

*Prediction* of *Clog*P *for compounds*

The Clog*P* value roughly corresponds to actual hydrophobicity. The Clog*P* values of compounds used in this study were calculated from the chemical structures using the computer software Chem Bio Office Ultra 2008 (PerkinElmer, Cambridge, USA).

## RESULTS AND DISCUSSION

*Inhibitory effects of cinnamic acid derivatives on HCV replication*

To examine effective inhibitory effects of cinnamic acid derivatives, we screened 14 de-

rivatives using HCV replicon cells. Each compound was added at various concentrations to the culture medium. HCV replicon RNA basically codes HCV 5'UTR (IRES), luciferase gene, EMCV IRES, HCV nonstructural gene, and HCV 3'UTR and autonomously replicated in the permitted cells. Thus, luciferase activity corresponded to HCV replication. Luciferase assays were carried out at 72 h post-treatment to determine the half maximal effective concentration ($EC_{50}$). Treatment with 1,2-dihydroxybenzene, which is called pyrocatechol, inhibited the viral replication with an $EC_{50}$ value of 13.1 ± 0.4 $\mu$M (Fig. 1). $EC_{50}$ values of compounds that lack the hydroxyl group at R1 (Compounds J to N) are markedly lower than those of compounds with the catechol ring (Compounds A-I) (Table I and Fig. 2), suggesting that the catechol ring is essential for antiviral activity of cinnamic acid derivatives. Compound F was identified as the most effective antiviral compound among the compounds used in this study (Table I and Fig. 3). However, the $EC_{50}$ value of compound F was slightly higher than that of compound 6, which

**A**



$EC_{50} = 7.5 \pm 0.5$ μM

**B**



$EC_{50} = 9.5 \pm 0.2$ μM
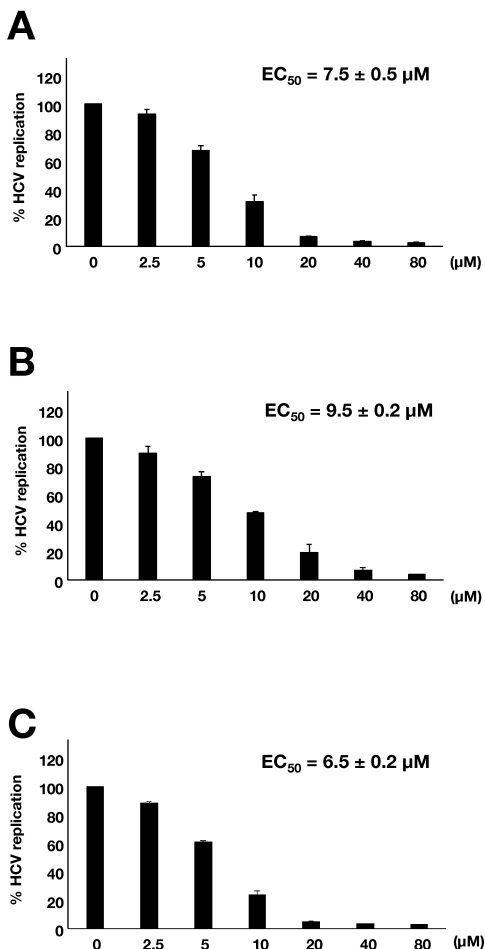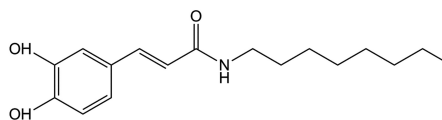
**C**



$EC_{50} = 6.5 \pm 0.2$ μM

Fig. 2. The antiviral activity and the cytotoxicity of compounds D, E, and F
The replicon cells were treated with an indicated concentration of compounds D (A), E (B), and F (C) as described in Materials and Methods. $CC_{50}$ values of compounds D, E, and F were $37.1 \pm 1.6$ μM, $53.4 \pm 6.5$ μM, and $28.2 \pm 1.3$ μM, respectively. The luciferase activity and cytotoxicity were evaluated. The data are representative of three independent experiments.
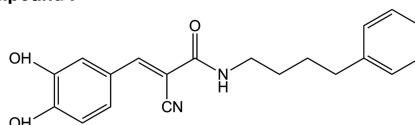
**Compound 6**



**Compound F**



Fig. 3. The structures of compounds 6 and F
We previously reported that compound 6 was reported to be antiviral agent for HCV [9]. Compound F was described in Table I.

because the CLog*P* value of compound 6 is higher than that of compound F (Table II). However, the possibility that other factors affect the antiviral activity of cinnamic acid-derived compounds could not be denied.

*Compound 6 impairs the production of HCVcc in culture supernatant*

We previously reported that compound 6 exhibits a significant inhibitory effect on the intracellular viral production and replication [9]. Production of infectious virus particles was impaired by treatment with compound 6 [9]. However, compound 6 included in the supernatant might affect viral titration. Next, we examined the effect of compound 6 on the amount of supernatant viral RNA. HCVcc-infected cells were seeded on the culture plate at 24 h before treatment. Fresh medium containing an indicated concentration of compound 6 was exchanged with the medium. These culture supernatants were harvested at 24 h post-treatment. Viral RNAs in culture supernatant were purified and quantified using qRT-PCR. The amount of viral

was previously reported as an anti-HCV agent by our group [9]. Compound 6 (Table II, Fig. 3) may be more effective as an antiviral agent against HCV and less toxic than compound F,

Table II.  $EC_{50}$ and $CC_{50}$ values of compounds 6 and F

| Compound | CLog$P$ | $EC_{50}$ ($\mu$M) | $CC_{50}$ ($\mu$M) | SI |
|----------|---------|-----------|-----------|-----|
| Compound 6 | 4.24 | 4.2 ± 0.8 | 201.8 ± 3.8 | 48.0 |
| Compound F | 2.97 | 6.5 ± 0.2 | 28.2 ± 1.3 | 4.2 |

The data of compound 6 were cited from reference[9].
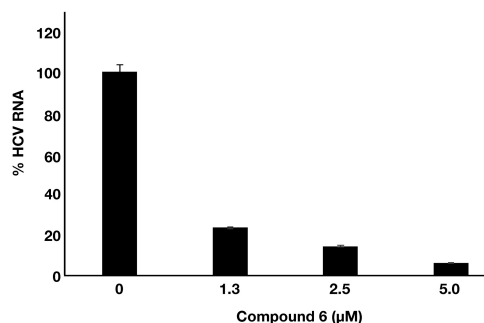SI indicates selectivity index.



Fig. 4.  Effect of compound 6 on HCVcc-infected cells. HCVcc-infected cells were seeded on 6-well plates at a density of $4.0 \times 10^5$ cells per well. After 24 h incubation, these cells were treated with an indicated concentration of compound 6. Viral RNAs were isolated 24 h post-treatment and were quantified by qRT-PCR. The data are representative of three independent experiments.

RNA in the medium was significantly decreased by treatment with compound 6 (Fig. 4), suggesting that compound 6 impairs the production of viral particles due to viral replication.

*Compound 6 possesses a synergistic antiviral effect with HCV inhibitors*

The combination of compound 6 with telaprevir, daclatasvir, or IFN-$\alpha$2b was analyzed to determine the effect of compound 6 on the antiviral activity of a known anti-HCV agent. Synergistic antiviral effects (CI < 0.9) were observed in replicon cells treated with compound 6 and daclatasvir or IFN-$\alpha$2b (Fig. 5). When cells were treated with both compound 6 and daclatasvir,

CI values ranged from 0.55 ($EC_{90}$, 13,000: 1 = compound 6: daclatasvir) to 0.87 ($EC_{75}$, 50000: 1 = compound 6: daclatasvir). On the other hand, the combination resulted in CI values ranging from 0.52 ($EC_{90}$, 1: 1 = compound 6: IFN-$\alpha$2b) to 0.79 ($EC_{75}$, 1: 1 = compound 6: IFN-$\alpha$2b). A synergistic effect was not observed in the cells treated with compound 6 and telaprevir. These data suggest that compound 6 may enhance DAA therapy for hepatitis C.

In this study, we tested the anti-HCV activity of cinnamic acid derivatives to determine a chemical motif critical for antiviral activity (Fig. 1 and Table I). Our data suggest that catechol group is critical for the anti-HCV activity of cinnamic acid derivatives, although we could not find a more effective compound than compound 6 with regard to anti-HCV activity. In a previous report, compound 6 was identified as a potent HCV inhibitor, which is a synthetic cinnamic acid derivative derived from AG490[9]. Compound 6 suppresses HCV replication via induction of oxidative stress rather than inhibition of tyrosine kinase activity[9,19]. Compound 6 exhibited the similar antiviral effect on the replications of genotype 1b, 2a, 3a, and 4a replicons[9]. Compound 6 may suppress the HCV replication regardless of viral genotype and/or sensitivity against current DAAs. The further study will be needed to clarify the spectrum of antiviral activity for compound 6. In this study, we tested the antiviral activity of compound 6 on HCVcc in the culture medium and combinations of compound 6 with
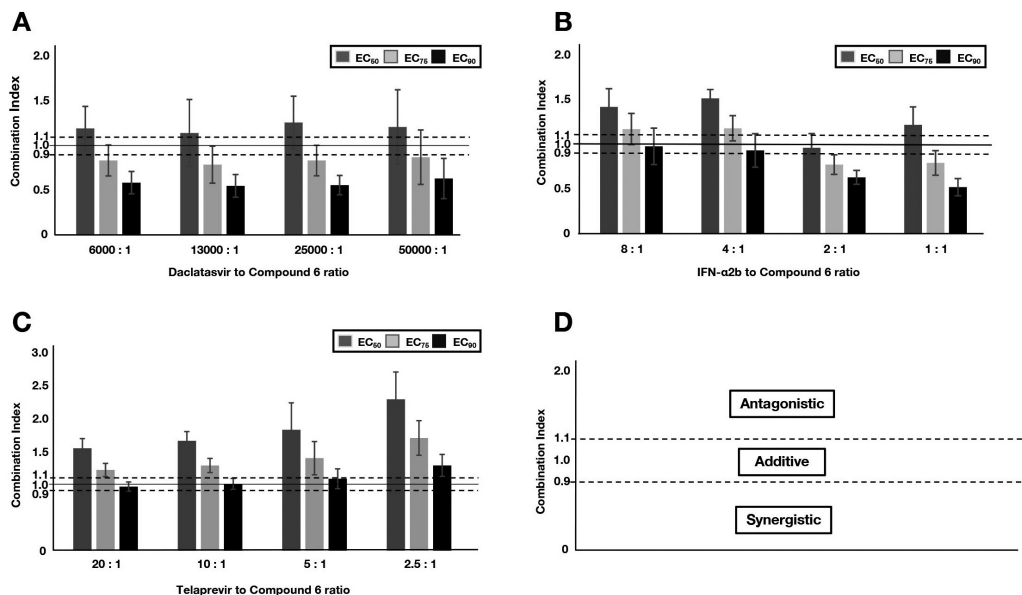
Fig. 5. Combination Index of compound 6 with daclatasvir, IFN-$\alpha$2b, or telaprevir. (A) The replicon cells were treated with compound 6 and daclatasvir. EC$_{50}$, EC$_{75}$, and EC$_{90}$ values were estimated at several concentrations of both agents. (B) The replicon cells were treated with compound 6 and IFN-$\alpha$2b. EC$_{50}$, EC$_{75}$, and EC$_{90}$ values were estimated at several concentrations of both agents. (C) The replicon cells were treated with compound 6 and telaprevir. EC$_{50}$, EC$_{75}$, and EC$_{90}$ values were estimated at several concentrations of both agents. (D) Schematic graph indicating antagonistic, additive, and synergistic effect. Combination index (CI) of > 1.1 indicates antagonistic effect, CI of 0.9-1.1 indicates additive effect, and CI of < 0.9 indicates synergistic effect.

clinically used anti-HCV agents. The antiviral effect of compound 6 on production of HCVcc in the culture medium was confirmed (Fig. 4). Compound 6 also showed synergistic antiviral effects with daclatasvir or IFN-$\alpha$2b (Fig. 5). Oxidative stress is associated with impairment of HCV replication as well as replication of other viruses[20]. An increase in nitric oxide down-regulated viral RNA synthesis on dengue virus type 2[21]. Induction of oxidative stress attenuated infectivity of influenza virus[22]. Therefore, induction of oxidative stress by treatment with compound 6 may affect not only replication of HCV but also other viral infections. Elevation of ROS can increase host genetic damage, but not influence much on in vivo heathy cells due

to high production of catalase. Ascorbate radical and hydrogen peroxide are produced by intravenous administration of high-dose vitamin C and exhibits cytotoxicity against tumor cells or IFN-treated cells but not healthy cells[23–25]. Thus, compound 6 may be non-toxic for heathy cells but toxic for virus-infected or tumor cells.

Development of an antiviral agent on the basis of compound 6 will advance establishment of a more effective HCV therapy in the future.

## CONCLUSION

In this study, our data suggest that the catechol group of a cinnamic acid derivative is essential for its anti-HCV activity and that compound 6

possesses synergistic anti-HCV effects with daclatasvir and IFN-$\alpha$2b. Induction of oxidative stress by a newly developed antiviral may lead to a novel therapeutic strategy for HCV therapy.

### REFERENCES

1) WHO: Global hepatitis reports: World Health Organization, 2017.
2) Asselah T, Boyer N, Saadoun D, Martinot-Peignoux M, Marcellin P: Direct-acting antivirals for the treatment of hepatitis C virus infection: optimizing current IFN-free treatment and future perspectives. Liver Int, 36 Suppl 1: 47–57, 2016.
3) Panel AIHG: Hepatitis C guidance: AASLD-IDSA recommendations for testing, managing, and treating adults infected with hepatitis C virus. Hepatology, 62: 932–954, 2015.
4) Adisakwattana S: Cinnamic Acid and Its Derivatives: Mechanisms for Prevention and Management of Diabetes and Its Complications. Nutrients, 9, 2017
5) De P, Baltas M, Bedos-Belval F: Cinnamic acid derivatives as anticancer agents-a review. Curr Med Chem, 18: 1672–1703, 2011.
6) Korosec B, Sova M, Turk S, Krasevec N, Novak M, *et al.*: Antifungal activity of cinnamic acid derivatives involves inhibition of benzoate 4-hydroxylase (CYP53). J Appl Microbiol, 116: 955–966, 2014.
7) Mielecki M, Lesyng B: Cinnamic Acid Derivatives as Inhibitors of Oncogenic Protein Kinases—Structure, Mechanisms and Biomedical Effects. Curr Med Chem, 23: 954–982, 2016.
8) Sova M: Antioxidant and antimicrobial activities of cinnamic acid derivatives. Mini Rev Med Chem, 12: 749–767, 2012.
9) Amano R, Yamashita A, Kasai H, Hori T, Miyasato S, *et al.*: Cinnamic acid derivatives inhibit hepatitis C virus replication via the induction of oxidative stress. Antiviral Research, 145: 123–130, 2017.
10) Okamoto T, Nishimura Y, Ichimura T, Suzuki K, Miyamura T, *et al.*: Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J, 25: 5015–5025, 2006.
11) Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, *et al.*: Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med, 11: 791–796, 2005.
12) Saeed M, Andreo U, Chung HY, Espiritu C, Branch AD, *et al.*: SEC14L2 enables pan-genotype HCV replication in cell culture. Nature, 524: 471–475, 2015.
13) Saeed M, Scheel TK, Gottwein JM, Marukian S, Dustin LB, *et al.*: Efficient replication of genotype 3a and 4a hepatitis C virus replicons in human hepatoma cells. Antimicrob Agents Chemother, 56: 5365–5373, 2012.
14) Kuo YH, Su MJ: Preparation of catechol derivatives for treatment of diabetes and ischemic diseases. PCT Int Appl. WO 2008028314 A1 20080313, 2008.
15) McCluskey A, Robinson PJ, Hill T, Scott JL, Edwards JK: Green chemistry approaches to the Knoevenagel condensation: comparison of ethanol, water and solvent free (dry grind) approaches. Tetrahedron Lett, 43: 3117–3120, 2005.
16) Jones JJ, Batoy SM, Wilkins CL, Liyanage R, Lay JO, Jr.: Ionic liquid matrix-induced metastable decay of peptides and oligonucleotides and stabilization of phospholipids in MALDI FTMS analyses. J Am Soc Mass Spectrom, 16: 2000–2008, 2005.
17) Fulop A, Porada MB, Marsching C, Blott H, Meyer B, *et al.*: 4-Phenyl-alpha-cyanocinnamic acid amide: screening for a negative ion matrix for MALDI-MS imaging of multiple lipid classes. Anal Chem, 85: 9156–9163, 2013.
18) Wells G, Seaton A, Stevens MF: Structural studies on bioactive compounds. 32. Oxidation of tyrphostin protein tyrosine kinase inhibitors with hypervalent iodine reagents. J Med Chem, 43: 1550–1562, 2000.
19) McCartney EM, Helbig KJ, Narayana SK, Eyre NS, Aloia AL, *et al.*: Signal transducer and activator of transcription 3 is a proviral host factor for hepatitis C virus. Hepatology, 58: 1558–1568, 2013.

20) Choi J, Lee KJ, Zheng Y, Yamaga AK, Lai MM, *et al.*: Reactive oxygen species suppress hepatitis C virus RNA replication in human hepatoma cells. Hepatology, 39: 81–89, 2004.

21) Takhampunya R, Padmanabhan R, Ubol S: Antiviral action of nitric oxide on dengue virus type 2 replication. J Gen Virol, 87: 3003–3011, 2006.

22) Sakudo A, Misawa T, Shimizu N, Imanishi Y: N(2) gas plasma inactivates influenza virus mediated by oxidative stress. Front Biosci (Elite Ed), 6: 69–79, 2014.

23) Kim HJ, Kim CH, Ryu JH, Kim MJ, Park CY, *et al.*: Reactive oxygen species induce antiviral innate immune response through IFN-lambda regulation in human nasal epithelial cells. Am J Respir Cell Mol Biol, 49: 855–865, 2013.

24) Narayanan A, Amaya M, Voss K, Chung M, Benedict A, *et al.*: Reactive oxygen species activate NFkappaB (p65) and p53 and induce apoptosis in RVFV infected liver cells. Virology, 449: 270–286, 2014.

25) Shatzer AN, Espey MG, Chavez M, Tu H, Levine M, *et al.*: Ascorbic acid kills Epstein-Barr virus positive Burkitt lymphoma cells and Epstein-Barr virus transformed B-cells in vitro, but not in vivo. Leuk Lymphoma, 54: 1069–1078, 2013.