



Anti-viral activity of compounds from *Agrimonia pilosa* and *Galla rhois* extract mixture

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ABSTRACT

Hepatitis C virus (HCV) infection is a significant health problem, with a worldwide prevalence of about 170 million. Recently, the development of direct acting antiviral (DAA) as a therapeutic agent for HCV has been rapidly increasing. However, DAA has a side effect and is costly. Therefore, it is still necessary to develop a therapeutic agent to treat HCV infection using products.

Agrimonia pilosa (AP) and *Galla rhois* (RG) are traditional medicines and are known to display therapeutic activity on various diseases. Notably, they have been reported to have an anti-viral effect on HBV and influenza virus infections. It is expected that anti-viral activity will increase when two extracts are mixed.

To investigate their anti-viral activity, the expression level of HCV Core 1b and NS5A was measured. Remarkably, AP, RG, and their mixed compound (APRG64) strongly inhibited the expression of viral proteins, which led us to identify their metabolites. A total of 14 metabolites were identified using liquid chromatography mass spectrometry (LC-MS). These metabolites were evaluated for their anti-HCV activity to identify active ingredients.

In conclusion, our results unveiled that anti-HCV activity of *Agrimonia pilosa* and *Galla rhois* extract mixture could lead to the development of a novel therapy for HCV infection.

1. Introduction

Hepatitis C virus (HCV) is one of the main causes of acute hepatitis, chronic infection, liver cirrhosis, and hepatocellular carcinoma. HCV is a positive-sense, single-stranded RNA virus in the flaviviridae family. It encodes a polyprotein that is cleaved by host and viral proteases to yield ten mature viral proteins, which are the core proteins such as envelope proteins E1 and E2, THE p7 ion channel protein, and non-structural (NS) proteins NS2, NS3, NS4A, NS5A and NS5B [1,2]. The ability of HCV to establish chronic infection in most of the infected patients is in part due to its regulation of critical signaling pathways in hepatocytes and its evasion of host innate immune responses [3].

The standard treatment for chronic hepatitis C is a combination of Peg-interferon (Peg-IFN) and ribavirin. When the two substances were

co-administered in liver transplant recipients, the virus suppression effect was increased by 10–50% [12–15]. However, sensitivity to continuous viral suppression was low and about 20% of patients discontinued the treatment due to side effects. Ribavirin remains a component of several direct-acting antiviral (DAA). Ribavirin was known to directly cause toxicity to red blood cells, leading to hemolytic anemia. Therefore, ribavirin is not retained at its initial capacity to affect the persistence of the viral response [4]. Peg-IFN can be used regardless of genotype of HCV because it indirectly suppresses the virus [5,6]. Therefore, it is required to develop novel therapeutic agents that can replace ribavirin and Peg-IFN.

Agrimonia pilosa, commonly known as agrimony, and the *Agrimonia* species have been reported to possess therapeutic effects on diverse diseases including anti-viral activity [7,8]. *Galla rhois*, the gall

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produced by the aphid *Schlechtendalia chinensis* (Bell), has been used as a traditional medicine for the treatment of diarrhea, excessive sweating, bleeding, and chronic coughs. Recently, *Galla rhois* was reported to have strong anti-viral and anti-biotic effects [9]. These results led us to investigate the anti-HCV effects of *Agrimonia pilosa* and *Galla rhois* extracts mixture. We found that a mixture of *Agrimonia pilosa* and *Galla rhois* extracts (APRG64) inhibited expression of HCV Core 1b and NS5A proteins. Furthermore, 14 metabolites that were identified from APRG64 using liquid chromatography mass spectrometry (LC-MS) were evaluated for anti-HCV activities.

2. Materials and methods

2.1. Plant extraction

The leaves of *Agrimonia pilosa* and *Galla rhois* were purchased from BioKorea Co., Ltd in Seoul, Korea, and the voucher specimen (BMRI-AP-1601, BMRI-RG-1602) was deposited at the Laboratory of Biomedical institute, Kyung Hee University, Yongin, Korea.

The dried samples (20 kg) were extracted with 50% EtOH at $80 \pm 2^\circ\text{C}$ for 6 h and then filtered. The ethanol extracts were concentrated using a rotary evaporator to lyophilize. Final product yields were 1.57 kg (*A. pilosa*) and 11.59 kg (*G. rhois*). The mixture of AP and RG was composed of the two extracts at the ratio of 6:4, respectively. All samples were stored at 4°C until use.

2.2. Metabolites profiling of APRG64 using HPLC-QTOF/MS

HPLC was performed using UltiMate™ 3000 Rapid Separation Binary System (Thermo fisher scientific Inc., Waltham, MA, USA) and Kinetex F5 C18 column (2.6 μm , 2.1×100 mm). The column oven was maintained at 25°C , and the mobile phases included solvent A [0.1% formic acid in water (v/v)] and solvent B [0.1% formic acid in acetonitrile (v/v)]. The elution gradient was as follows: 0–2 min, B 5%; 2–8 min, B 5–20%; 8–16 min, B 20%; 16–28 min, B 20–35%; 28–32 min, B 35–100%; and 32–35 min, B 100%. The flow rate was 250 $\mu\text{L}/\text{min}$, and the injection volume was 5 μL for each run.

MS analysis was performed using a SCIEX Triple TOF 5600 (SCIEX, Framingham, MA, USA) operating in a positive ion mode. The mass spectrometers performed alternating high- and low-energy scans, known as the MS^E acquisition mode. The operating parameters were set as follows: cone voltage 40 V; capillary 3.0 kV; source temperature 500°C ; cone gas flow 30 L/h; and desolvation gas flow at 800 L/h. Data were collected between 100 and 1000 m/z .

2.3. Cell culture

Huh 7.5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, WELGENE, Daegu, Korea) supplemented with 10% Fetal Bovine Serum (FBS, GE Healthcare Life Sciences) and 1% Penicillin-Streptomycin (PS, WELGENE, Daegu, Korea). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 .

2.4. HCV RNA preparation

2.4.1. Transformation

Competent cells for transformation were performed according to the manufacturer's instruction (ECOS 101, Yeastern Biotech Co., Ltd, Taipei, Taiwan). Briefly, 1 μL of plasmid DNA was mixed with 50 μL of cells and then incubated on ice for 5 min. The incubated cells were applied onto an ampicillin-containing agar plate followed by incubation in a 37°C incubator until bacterial colonies appeared.

2.4.2. Midiprep

Midiprep was performed using PureLink™ HiPure Plasmid DNA Purification Kits (Invitrogen, CA, USA). The plasmid DNA was dissolved

in an appropriate volume of TE buffer whose concentration and purity were determined using a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher, Waltham MA, USA). The 1 μg of obtained DNA applied to an agarose gel containing GelRed® Nucleic Acid Gel Stain (Biotium, CA, USA) in Mupid-2 Plus electrophoresis system (ADVANCE).

2.4.3. DNA linearization

The JFH-1 plasmid was kindly provided by Dr. Takaji Wakita (National Institute of Infectious Diseases, Japan). The plasmid was digested with Xba I (New England Biolabs, NEB, MA, USA) at 37°C overnight, followed by incubation with Mungbean nuclease (New England Biolabs, NEB, MA, USA) for 12 min. The linearized plasmid DNA was extracted twice by phenol:chloroform:isoamyl alcohol and precipitated with NaAc pH 5.2 and 100% EtOH. After washing once with 70% EtOH, the concentration and purity were determined using the spectrophotometer.

2.4.4. In vitro transcription

The *in vitro* transcription was performed using the MEGAScript® kit (Life Technology, CA, USA) according to the manufacturer's instruction. After the transcription, the extracted RNA was precipitated with the same volume of isopropanol. After sufficiently dissolving the precipitated RNA pellet with RNase free water, the concentration and purity were confirmed by the spectrophotometer.

2.5. Transfection

The Neon® transfection system (Invitrogen, CA, USA) was used for electroporation. Briefly, Huh 7.5 cells were resuspended with 100 μL R buffer and the extracted RNA was added in. After adding the cell mixture into 100 μL Neon® tip, shock (1400 V-20 ms-1 pulse) was applied. The shocked cells were then resuspended with antibiotics free media and incubated at 37°C in a humidified atmosphere with 5% CO_2 .

3. Results and discussion

3.1. Anti-HCV activity of APRG64

To address whether our extracts (AP, RG, APRG64) exhibit anti-HCV activity, Huh 7.5 cells were transfected with full length HCV RNAs transcribed from pJFH-1 plasmid. Two days later, Huh 7.5 cells were treated with each extract for two additional days. Since the expression levels of HCV Core 1b and NS5A are indicative of replication of HCV [10,11], the expression levels of these proteins were analyzed by Western blotting. Remarkably, AP and RG strongly inhibited the expression of Core 1b and NS5A (Fig. 1). These results led us to speculate that the mixture of these extracts could be more efficient to control HCV. We used the mixture of AP and RG (APRG64) to test its antiviral

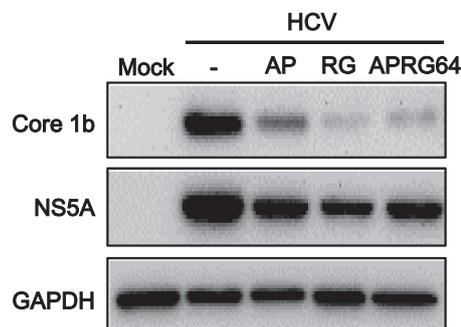
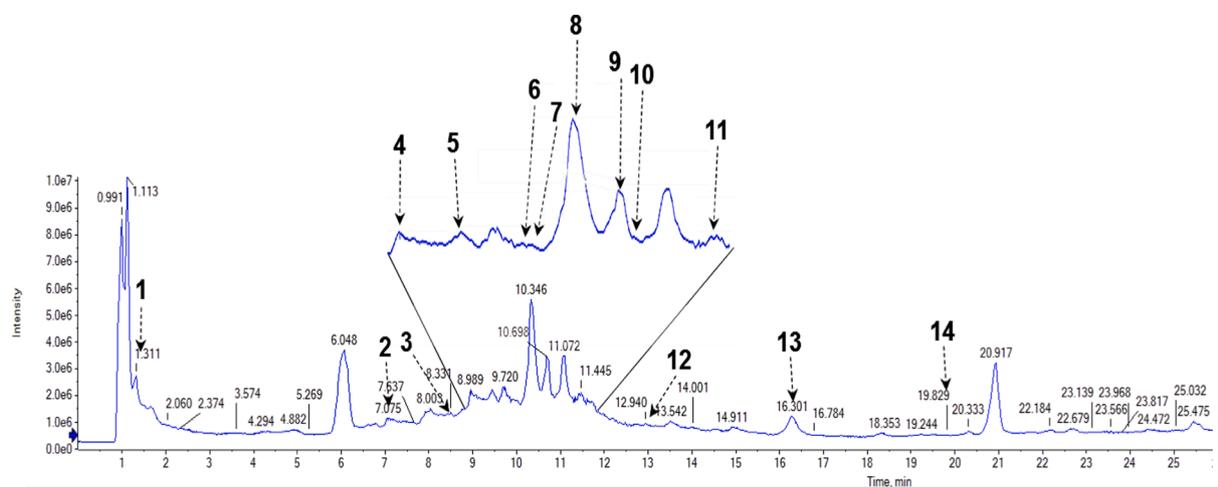


Fig. 1. Anti-HCV activity of AP, RG, and APRG64. Huh7.5 cells that were untransfected (Mock) or transfected with HCV RNAs were either untreated or treated with extracts of AP (5 $\mu\text{g}/\text{mL}$), RG (5 $\mu\text{g}/\text{mL}$), and APRG64 (5 $\mu\text{g}/\text{mL}$) for 48 hrs. Cell lysates were prepared for the western blot analysis to detect HCV Core 1b and NS5A. GAPDH was used as a loading control.



| No. | RT (min) | Metabolites | Molecular Formula | Observed Mass (Da) | Expected Mass (Da) | Mass Accuracy (ppm) |
|-----|----------|------------------------------|---|--------------------|--------------------|---------------------|
| 1 | 1.14 | procyanidin B2 | C ₃₀ H ₂₆ O ₁₂ + H | 579.1488 | 579.1502 | 2.5 |
| 2 | 7.12 | dihydroquercetin | C ₁₅ H ₁₂ O ₇ + H | 305.0659 | 305.0661 | 0.8 |
| 3 | 8.48 | coumarin | C ₉ H ₆ O ₂ + H | 147.0437 | 147.0446 | 6.3 |
| 4 | 9.21 | rutin | C ₂₇ H ₃₀ O ₁₆ + H | 611.1605 | 611.1612 | 1.2 |
| 5 | 9.44 | hyperin | C ₂₁ H ₂₀ O ₁₂ + H | 465.1030 | 465.1033 | 0.7 |
| 6 | 10.25 | luteolin | C ₁₅ H ₁₀ O ₆ + H | 287.0552 | 287.0555 | 1.1 |
| 7 | 10.30 | astragalín | C ₂₁ H ₂₀ O ₁₁ + H | 449.1073 | 449.1084 | 2.5 |
| 8 | 10.33 | quercetin | C ₁₅ H ₁₀ O ₇ + H | 303.0498 | 303.0505 | 2.4 |
| 9 | 10.70 | apigenin 7-O-β-D-glucuronide | C ₂₁ H ₁₈ O ₁₁ + H | 447.0923 | 447.0927 | 1.0 |
| 10 | 10.76 | baicalin | C ₂₂ H ₂₀ O ₁₀ + H | 447.0936 | 447.0927 | -2.0 |
| 11 | 11.46 | afzelin | C ₂₁ H ₂₀ O ₁₀ + H | 433.1126 | 443.1135 | 2.1 |
| 12 | 12.96 | nicotiflorin | C ₂₇ H ₃₀ O ₁₅ + H | 595.1446 | 595.1663 | 36.5 |
| 13 | 16.28 | tiliroside | C ₃₀ H ₂₆ O ₁₃ + H | 595.1445 | 595.1451 | 1.1 |
| 14 | 19.83 | apigenin | C ₁₅ H ₁₀ O ₅ + H | 271.0602 | 271.0606 | 1.6 |

Fig. 2. HPLC-QTOF/MS chromatograms of APRG64, and mass data in total ion chromatograms (TIC) scan mode. The peaks of 1–14 were identified to be 14 metabolites as listed in the Table. Analysis was carried out on a Kinetex F5 C18 column (2.6 μm, 2.1 × 100 mm) with gradient elution using solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The elution gradients were as follows: 0–6 min, B 5%; 6–8 min, B 5–15%; 8–16 min, B 15–20%; 16–27 min, B 20–30%; 27–32 min, B 30–100%; and 30–34 min, B 100%. The flow rate was 250 μL/min, and the injection volume was 5 μL for each run. The detection was carried out by HPLC-QTOF/MS. Mass detector settings were as follows: cone voltage 40 V; capillary 3.0 kV; source temperature 500 °C; cone gas flow 30 L/h; and desolvation gas flow at 800 L/h.

effect on HCV replication. Indeed, APRG64 potently inhibited expression of HCV proteins. Although APRG64 is composed of AP and RG at the ratio of 6:4 respectively, its inhibitory effect was much stronger than AP and also comparable to RG. Therefore, this result indicated that AP and RG have a combination effect to inhibit HCV replication (Fig. 1).

3.2. Profiling of various metabolites in APRG64

Since APRG64 displayed strong inhibitory effects on HCV protein expression, we decided to identify their active ingredients using a LC-MS. Its metabolites were separated within 1–26 min at a flow rate of 250 μL/min by using the HPLC system with a Kinetex F5 C18 column.

The total ion chromatograms (TIC) of the APRG64 are shown in Fig. 2. APRG64 was analyzed using HPLC-QTOF/MS; each piece of data was then processed using the Analyst TF1.7 (SCIEX, Framingham, MA, USA) software. Information about the metabolite's name, molecular formula, retention time (RT), observed mass value, and mass accuracy were listed for the 14 metabolites (Fig. 2). The peaks were selected based upon the shared traits of having value higher than 0.95 in the Analyst library as well as a mass accuracy of each peak smaller than 40 ppm.

3.3. Anti-HCV activity of APRG64 metabolites

We first determined the concentration of the isolated metabolites that did not display the cytotoxicity. Huh 7.5 cells were treated with

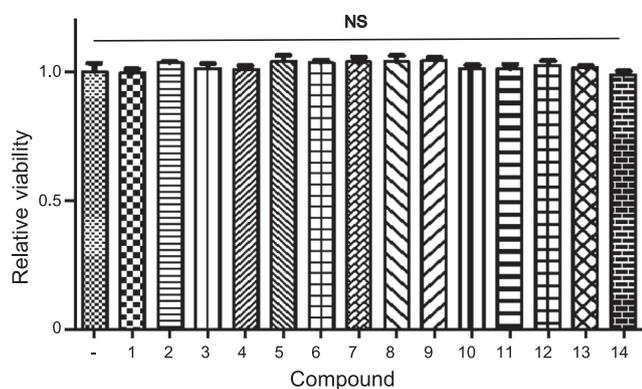


Fig. 3. Cytotoxicity of APRG64 metabolites. Huh7.5 cells were treated with Procyanidin B2 (1, 5 $\mu\text{g}/\text{mL}$), Dihydroquercetin (2, 5 $\mu\text{g}/\text{mL}$), Coumarin (3, 50 $\mu\text{g}/\text{mL}$), Rutin (4, 5 $\mu\text{g}/\text{mL}$), Hyperin (5, 5 $\mu\text{g}/\text{mL}$), Luteolin (6, 2.5 $\mu\text{g}/\text{mL}$), Astragalin (7, 50 $\mu\text{g}/\text{mL}$), Quercetin (8, 2.5 $\mu\text{g}/\text{mL}$), Apigenin-7-o-glucuronide (9, 50 $\mu\text{g}/\text{mL}$), Baicalin (10, 50 $\mu\text{g}/\text{mL}$), Afzelin (11, 50 $\mu\text{g}/\text{mL}$), Nicotiflorin (12, 50 $\mu\text{g}/\text{mL}$), Tiliroside (13, 50 $\mu\text{g}/\text{mL}$), and Apigenin (14, 5 $\mu\text{g}/\text{mL}$) for 24 hrs. Cytotoxicity was determined by a trypan blue exclusion assay. NS, not significant.

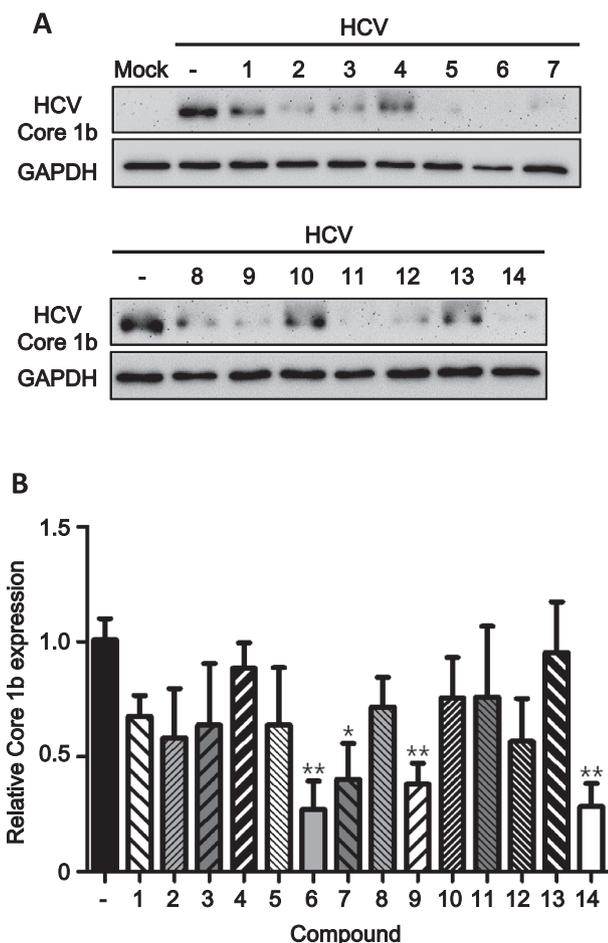


Fig. 4. Inhibitory effects of APRG64 metabolites on the expression of HCV Core 1b. Untransfected (Mock) or transfected Huh7.5 cells with HCV RNAs were untreated or treated with 14 APRG64 metabolites. (A) Expression of HCV Core 1b was analyzed by the western blot analysis. GAPDH was used as a loading control. (B) Band intensity of HCV Core 1b protein was quantified by Image J software (NIH) and relative expressions to GAPDH are shown. *, $p \leq 0.05$; **, $p \leq 0.01$.

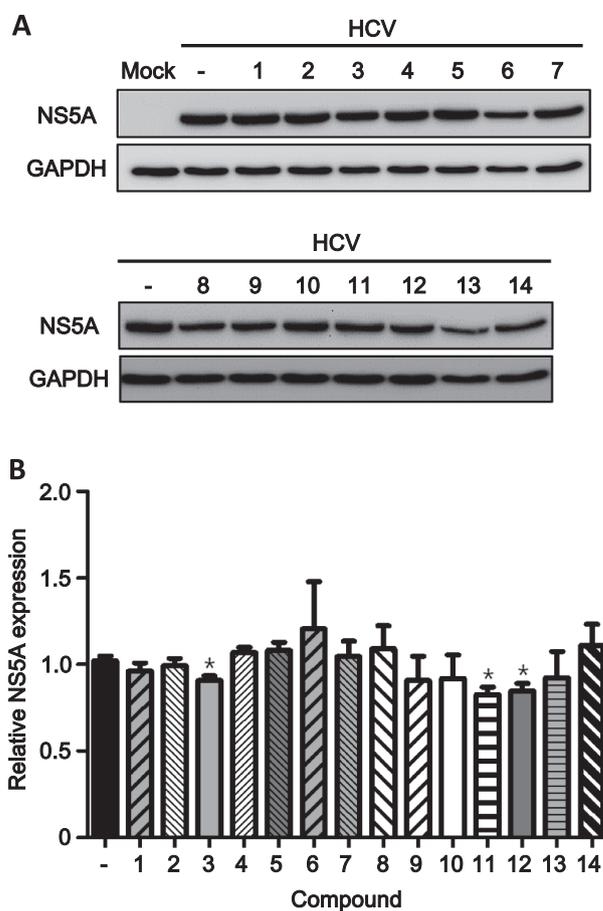


Fig. 5. Inhibitory effects of APRG64 metabolites on the expression of HCV NS5A. Huh7.5 cells that were either untransfected (Mock) or transfected with HCV RNAs were untreated or treated with 14 APRG64 metabolites. (A) Expression of HCV NS5A was analyzed by the western blot analysis. GAPDH was used as a loading control. (B) Band intensity of HCV Core 1b protein was quantified by Image J software and relative expressions to GAPDH are shown. *, $p \leq 0.05$.

various concentrations of each metabolite to analyze cytotoxicity by a trypan blue exclusion assay. Optimal concentration of each metabolite that did not show the significant cytotoxicity was shown in Fig. 3. These concentrations were used for subsequent experiments. Next, we addressed whether these compounds exhibited anti-HCV activity. Interestingly, all compounds inhibited the expression of HCV Core 1b protein when compared to an untreated control. Of note, hyperin, luteolin, astragalin, afzelin and apigenin displayed relatively strong inhibitory activity as compared to other compounds (Fig. 4A and B). Additionally, we analyzed whether these metabolites affected the expression of HCV NS5A protein. Similar to the results in Fig. 4, these metabolites also showed notable inhibitory effects on the expression of NS5A (Fig. 5A and B). Among compounds, luteolin and tiliroside displayed relatively strong inhibitory activity on HCV NS5A expression. Thus, we could conclude that these fractions exhibit strong inhibitory activity on expression of HCV proteins.

All 14 metabolites were isolated from *Agrimonia pilosa* and *Galla rhois*. Apigenin-7-O- β -D-glucuronide, procyanidin B2, tiliroside and nicotiflorin were isolated and observed only in *Agrimonia* species. Our results indicated that the anti-HCV effects of hyperin, luteolin, astragalin, afzelin, and apigenin that were separated and observable in both *Agrimonia pilosa* and *Galla rhois* were excellent. Therefore, it is expected that when two extracts are mixed, the anti-viral activity will be increased and the virus sensitivity will be maintained rather than using a single extract.

4. Conclusion

In this study, it was confirmed that *Galla rhois* extract was superior to *Agrimonia pilosa* extract in suppressing HCV Core 1b and NS5A expression. The metabolites in APRG64 were identified using UPLC-QTOF/MS analysis. Identification of the peak was carried out through comparison of mass data for each peak with those in the library Analyst. The metabolites were selected according to the shared traits of having value higher than 0.95 in the library as well as a mass accuracy of each peak smaller than 40 ppm.

LC-MS analysis confirmed HCV Core 1b and NS5A expression regulation using the identified components and observed the component with anti-viral activity.

We confirmed the possibility of developing a novel HCV treatment strategy using natural product extracts.

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2019.103320>.

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