Hepatitis C virus (HCV) infection is a major cause of chronic liver disease such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. While searching for new natural anti-HCV agents in agricultural products, we found a potent inhibitor of HCV RNA expression in extracts of blueberry leaves when examined in an HCV subgenomic replicon cell culture system. This activity was observed in a methanol extract fraction of blueberry leaves and was purified by repeated fractionations in reversed-phase high-performance liquid chromatography. The final purified fraction showed a 63-fold increase in specific activity compared with the initial methanol extracts and was composed only of carbon, hydrogen, and oxygen. Liquid chromatography/mass-ion trap-time of flight analysis and butanol-HCl hydrolysis analysis of the purified fraction revealed that the blueberry leaf-derived inhibitor was proanthocyanidin. Furthermore, structural analysis using acid thiolysis indicated that the mean degree of polymerization of the purified proanthocyanidin was 7.7, consisting predominantly of epicatechin. Proanthocyanidin with a polymerization degree of 8 to 9 showed the greatest potency at inhibiting the expression of subgenomic HCV RNA. Purified proanthocyanidin showed dose-dependent inhibition of expression of the neomycin-resistant gene and the NS-3 protein gene in the HCV subgenome in replicon cells. While characterizing the mechanism by which proanthocyanidin inhibited HCV subgenome expression, we found that heterogeneous nuclear ribonucleoprotein A2/B1 showed affinity to blueberry leaf-derived proanthocyanidin and was indispensable for HCV subgenome expression in replicon cells. These data suggest that proanthocyanidin isolated from blueberry leaves may have potential usefulness as an anti-HCV compound by inhibiting viral replication.

This study was supported by a grant from the Collaboration of Regional Entities for the Advancement of Technological Excellence (CREATE) from Japan Science and Technology Agency.

1 To whom correspondence should be addressed: Section of Oncopathology and Regenerative Biology, Dept. of Pathology, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan. Tel.: 81-985-85-2809; Fax: 81-985-85-6003; E-mail: mejina@fc.miyazaki-u.ac.jp.

2 The abbreviations used are: HCV, hepatitis C virus; hnRNP, heterogeneous nuclear ribonucleoprotein; HPLC, high-performance liquid chromatography; IC50, concentration required for 50% inhibition; CC50, concentration required for 50% cytotoxicity; eIF3, eukaryotic translation initiation factor 3; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; IRES, internal ribosome entry site; DIGE, differential gel electrophoresis; PDI, photodiode array; EPMA, electron probe micro-analysis; LC/MS-IT-TOF, liquid chromatography/mass spectrometry-ion trap-time of flight; APCLI, atmospheric pressure chemical ionization; mDP, mean degree of polymerization; Cd50, concentration required for 50% inhibition; CCd50, concentration required for 50% cytotoxicity; eIF3, eukaryotic translation initiation factor 3; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; IRES, internal ribosome entry site; DIGE, differential gel electrophoresis.

Proanthocyanidin from Blueberry Leaves Suppresses Expression of Subgenomic Hepatitis C Virus RNA*[S]

Received for publication, April 6, 2009, and in revised form, June 12, 2009 Published, JBC Papers in Press, June 16, 2009, DOI 10.1074/jbc.M109.004945

Masahiko Takeshita*1, Yo-ichi Ishida*1, Ena Akamatsu*1, Yusuke Ohmori*1, Masayuki Sudo*1, Hirofumi Uto*1, Hirohito Tsubouchi*1, and Hiroaki Kataoka*2*1

From the 1Research Division, Minami Nippon Dairy Co-op Co., Ltd., Miyazaki 885-0073, the 2Miyazaki Prefectural Industrial Support Foundation, Miyazaki 880-0303, the 3Kamakura Research Laboratories, Chugai Pharmaceutical Co., Ltd., Kanagawa 247-8530, the 4Department of Digestive Disease and Life-style Related Disease, Health Research Human and Environmental Sciences, Kagoshima University, Graduate School of Medicine and Dental Sciences, Kagoshima 890-8520, and the 5Section of Oncopathology and Regenerative Biology, Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki 889-1692, Japan

* This study was supported by a grant from the Collaboration of Regional Entities for the Advancement of Technological Excellence (CREATE) from Japan Science and Technology Agency.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.
Blueberry Leaf Proanthocyanidin Suppresses HCV

In this study, extracts of rabbit-eye blueberry leaves were used in an effort to purify and identify the compound responsible for inhibition of the expression of subgenomic HCV RNA. We identified oligomeric proanthocyanidin with mean degree of polymerization (mDP) around eight as an inhibitor of HCV subgenome expression. We also analyzed cellular proteins that have affinity to the oligomeric proanthocyanidin in HCV replicon cells and identified heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 as one of candidate proteins involved in the proanthocyanidin-mediated inhibition of HCV subgenome expression.

**EXPERIMENTAL PROCEDURES**

**Extraction of Blueberry Leaves**—A lyophilized powder made from leaves of rabbit-eye blueberry (V. virgatum Aiton) was provided by Unkai Shuzo Co., Ltd. (Miyazaki, Japan). One gram of the lyophilized powder was extracted with 100 ml of methanol at room temperature with shaking for 15 min, and the supernatant was passed through filter paper (filter paper No.2, Toyo, Tokyo, Japan). The methanol extract was then extracted with 100 ml of chloroform, followed by centrifugation (1750 × g for 10 min), and the resultant precipitate and supernatant were collected. The precipitate was dissolved in methanol, concentrated in vacuo, and lyophylized (CMW-ppt). The supernatant was mixed with 150 ml of distilled water and methanol to perform a liquid-liquid extraction, and the water layer was collected and mixed with 150 ml of chloroform to repeat the chloroform extraction. The water layer was concentrated and lyophylized (CMW-W). The chloroform layer was also concentrated and lyophylized (CMW-C). Most HCV subgenome-expression inhibitory activity was recovered in the CMW-W fraction.

**Preparative Fractionation by HPLC**—To separate the components in the CMW-W fraction processing inhibitory activity against HCV RNA expression, we performed HPLC (Prominence System, Shimadzu, Kyoto, Japan). Preliminary fractionation of CMW-W to confirm the elution pattern of HCV expression suppressive components was carried out on a reversed-phase column (Atlantis dC18, 4.6 mm × 150 mm, 3 μm, Waters, Milford, MA) at 40 °C with UV detection at 254 nm. A gradient consisting of eluant A (0.05% trifluoroacetic acid) and eluant B (acetonitrile) was applied at a flow rate of 0.7 ml/min as follows: 15–25% B linear from 0 to 12.5 min, 25–100% B linear from 12.5 to 17.5 min followed by washing 100% B from 17.5 to 25 min. For purification, the first HPLC fractionation was performed on a reversed-phase column (Atlantis T3, 4.6 mm × 150 mm, 3 μm, Waters). A gradient consisting of eluant A and eluant B (acetonitrile) was applied at a flow rate of 0.7 ml/min as follows: 30% B from 0 to 7.5 min, 30–100% B linear gradient from 7.5 to 12.5 min, followed by washing with 100% B from 12.5 to 20 min. The CMW-W fraction dissolved in 30 ml of methanol was injected, and the eluted fractions (2.1 to 18.0 min, total 26 fractions) were collected. The gradient program for the second fractionation was 20% B from 0 to 7.5 min, 20–100% B linear from 7.5 to 12.5 min, followed by washing with 100% B from 12.5 to 20 min. Fractionation of the eluate was the same as the first HPLC program. In the third HPLC fractionation, the eluant B was replaced by methanol and eluted with 40–65% B linear gradient from 0 to 12.5 min and 65–100% B linear gradient from 12.5 to 17.5 min. Fractions eluted from 2.2 to 17.5 min (total 26 fractions) were collected. In all experiments, suppressive activity of each fraction against HCV RNA expression was measured using replicon cells.

**HCV Replicon Cells and Replicon Assay**—The Huh-7/3-1 cell line carrying an HCV-replicon was used (10). The line was established from Huh-7 cells by stable transfection with subgenomic selectable RNA in which the encoding HCV structural proteins were replaced by the firefly luciferase gene, the internal ribosome entry site (IRES) of the Encephalomyocarditis virus and the neomycin phosphotransferase gene. With this HCV subgenome, the efficiency of subgenomic HCV expression could be estimated by measuring luciferase activity in the replicon cells. The HCV replicon cells were routinely grown in Dulbecco’s modified Eagle’s medium supplemented with Glutamax (Invitrogen), 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), and 500 μg/ml G418 (Invitrogen). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. For the HCV subgenome expression assay, the replicon cells in Dulbecco’s modified Eagle’s medium supplemented with Glutamax and 5% fetal bovine serum were seeded in 96-well plates (5000 cells/well) and incubated for 24 h. Then the cells were cultured with various concentrations of samples for 72 h. Quantification of the luciferase activity was performed using the Steady-Glo Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s instructions, and the luminescence was measured by DTX 800 Multimode Detector (Beckman Coulter, Fullerton, CA). The inhibitory activity was expressed as the concentration required for 50% inhibition (IC50). Specific activity was calculated as a reciprocal number of IC50 (1/IC50). Total activity was calculated by multiplying yielded weight by specific activity.

The cytotoxicity of the samples was measured by Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, 10 μl/well of Cell Counting Kit-8 reagent was added to the cells cultured in a 96-well plate, incubated at 37 °C for 60 min. The absorbance of each well was measured at 450 nm with a reference wavelength at 650 nm using an Emax Precision microplate reader (Molecular Devices Inc., Sunnyvale, CA). Cell viability was calculated as relative index of control cells, and effects of samples on cell viability were expressed as the concentration required for 50% cytotoxicity (CC50).

**Constitutive Analysis of Electron Probe Micro-analysis and Liquid Chromatography/Mass Spectrometry-ion Trap-time of Flight (LC/MS-IT-TOF)**—For electron probe micro-analysis (EPMA-1600, Shimadzu), the excitation voltage and the beam current were kept at 15 kV and at 100 nA, respectively. The diameter of the electron beam was 50 μm, and the sample was processed for carbon shadowing in advance.

Identification of the anti-HCV compound purified from blueberry leaves was done by HPLC-MSn fragmentation analyses. An HPLC System (Prominence System, Shimadzu) on a reversed-phase column (Atlantis dC18, 4.6 mm × 150 mm, 3 μm, Waters) was equipped with a photodiode array (PDA) detector scanning from 200 to 800 nm and mass spectrometry-ion trap-time of flight (MS-IT-TOF, Shimadzu).
Blueberry Leaf Proanthocyanidin Suppresses HCV

Preparation of Proanthocyanidin from Blueberry Leaves—To prepare proanthocyanidin from blueberry leaves, freeze-dried powder (100 g) was extracted with 1.2 liters of acetone for 10 min, and the supernatant was decanted. This procedure was repeated five times to remove the green pigment from the leaves, followed by washing in 1.2 liters of hexane for 30 min. The remaining residues were washed with ethyl acetate. The washed powder of leaves was extracted with 1.2 liters of methanol for 30 min, and the supernatant was filtered. This procedure was repeated four times, and the resulting crude methanol extracts were concentrated by rotary evaporator at 50 °C and lyophilized, finally resulting in ∼30 g of solid powder. The crude methanol extract (15 g) was then dissolved in 1.0 liter of 60% methanol and placed on a Sephadex LH-20 column (50 mm × 920 mm, Amersham Biosciences). Fractionation was performed using the following series of solvents: fraction I, 9.0 liters of 60% methanol (retrieved weight: 10.2 g); fraction II, 9.0 liters of 100% methanol (retrieval weight: 3.3 g); fraction III, 9.0 liters of 70% (v/v) acetone (retrieved weight: 1.3 g). The LC/MS-IT-TOF analyses of each fraction indicated that fraction I was primarily composed of quinic acid, chlorogenic acid, and flavonol glycosides such as rutin. Fraction II consisted of proanthocyanidin oligomers from tetramer to decamer as analyzed by thiolysis. Fraction III consisted of proanthocyanidin polymers that were decamers or greater. In each fraction, the eluate was divided into 28 subfractions/liter.

Northern Blot Analysis—Total RNAs from cultured replicon cells were prepared using mRNAeasy mini kits (Qiagen). RNAs were denatured at 65 °C for 15 min, cooled on ice, and then separated by 1% agarose-formaldehyde gel electrophoresis (2 μg/lane) and transferred to a positively charged nylon membrane (Hybond-N+, Amersham Biosciences). The membrane was hybridized with a biotinized probe of the neoynycin phosphotransferase gene. For detection of the bound probe, membranes were incubated with streptavidin-Alexa Fluor 680 conjugate (Invitrogen), and the bound fluorescence was detected by Odyssey Infrared Imaging System (LI-COR Biosciences). For internal control, β-actin mRNA-specific biotinized antisense RNA probe was used.

Western Blot Analysis—Cultured replicon cells were harvested, and total cellular proteins were extracted with Cel-Lytic-M (Sigma-Aldrich) containing 1% protease inhibitor mixture (Sigma-Aldrich). The samples were separated by SDS-PAGE using 10% gel under reducing conditions. The proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA).

The membrane was treated with a blocking buffer for near infrared fluorescent Western blotting (Rockland, Gilbertsville, PA). Primary antibodies used were anti-human hnRNP A2/B1, hnRNP K, hnRNP L, and hnRNP Q and anti-human β-actin antibodies (EF-67, D-6, A-11, 18E4, and I-19, respectively, Santa Cruz Biotechnology, Santa Cruz, CA), anti-human eukaryotic translation initiation factor 3 (eIF3) F, eIF3G eIF3H polyclonal antibodies (Novus Biologicals, Littleton, CO), and
Blueberry Leaf Proanthocyanidin Suppresses HCV

anti-HCV NS-3 polyclonal antibody (10). The labeled proteins were visualized with Alexa Fluor 680 anti-rabbit or anti-mouse IgG (Invitrogen) or IRDye™ 800CW anti-goat IgG (LI-COR Biosciences) and detected by using as Odyssey Infrared Imaging System.

Affinity Purification of Proanthocyanidin-binding Proteins—Purified blueberry leaf-derived proanthocyanidin or catechin was coupled with epoxy-activated Sepharose 6B (Amersham Biosciences) according to the manufacturer’s instructions. Approximately $5 \times 10^8$ HCV replicon cells were extracted with lysis buffer (50 mM sodium phosphate (pH 7.5), 1% CHAPS, 5 mM EDTA, 150 mM NaCl, and protease inhibitor mixture (Complete™, Roche Diagnostics, Mannheim, Germany)). The total protein extract (90 mg) was added to the coupled Sepharose beads (3 ml) and incubated at 4 °C overnight with gentle rotation. The beads were centrifuged (500 $\times g$) for 1 min, and the pellet was washed six times with the lysis buffer. The absorbed proteins were eluted by incubation in 2% SDS with 50 mM dithiothreitol at 100 °C for 10 min. The eluate was concentrated with an Amicon Ultra-4 Ultracel-5k (Millipore), and the solvent was replaced by the lysis buffer. Protein concentration was determined by the o-phthalaldehyde method using bovine serum albumin as the standard.

Fluorescent Two-dimensional DIGE—Fluorescent two-dimensional-DIGE was performed using fluorescent dyes, IC3-OSu and IC5-OSu (Dojindo Molecular Technologies), with a modification of the methods reported elsewhere (15, 16). Briefly, 10 $\mu$g of proteins per gel were precipitated using a two-dimensional clean-up kit (Bio-Rad) and then dissolved in 20 $\mu$l of sample buffer (10 mM sodium phosphate (pH 8.0), 7 M urea, 2 M thiourea, 3% CHAPS, and 1% Triton X-100). After addition of 400 pmol of IC3-OSu or IC5-OSu, proteins were incubated at 40 °C for 30 min. The labeling reaction was quenched by incubation with 400 $\mu$M lysine for 15 min, followed by addition of an equal volume of the sample buffer with 150 mM dithiothreitol, 0.4% Bio-Lyte 3–10 (Bio-Rad Laboratories), and 0.004% bromophenol blue. Two-dimensional gel electrophoresis was performed according to the manufacturer’s instructions (Bio-Rad). The mixed samples were applied to ReadyStrip IPG strips (pH 3–10 NL, 7 cm, Bio-Rad) for separation in the first dimension. The mixed samples were applied to ReadyStrip IPG strips (pH 3–10 NL, 7 cm, Bio-Rad) for separation in the first dimension. The mixed samples were applied to ReadyStrip IPG strips (pH 3–10 NL, 7 cm, Bio-Rad) for separation in the first dimension.

RESULTS

Purification of an Inhibitor of HCV Subgenome Expression from Blueberry Leaves—We screened 283 species of local agricultural products for their suppressive activity against the expression of subgenomic HCV RNA using an HCV replicon cell system, and found significant suppressive activity in the leaves of the blueberry (Vaccinium virgatum Aiton), peels of roots of Taro (Colocasia esculenta L.), and hulls of seeds of Japanese plum (Prunus mume Sieb. et Zucc.). Among them, extracts of blueberry leaves contained the highest total activities. Therefore, we purified a compound from blueberry leaves that inhibited expression of subgenomic HCV RNA in replicon cells. An overall purification scheme is shown in Fig. 1, and a summary of the purification steps is shown in Table 1. From 1000 mg of lyophilized powder from the leaves, 440 mg of methanol extracts was obtained. The IC_{50} value of the methanol extracts was 5.47 $\mu$g/ml. The inhibitory activity was recovered in the CMW-W fraction (284.2 mg), in which the IC_{50} value was 1.74 $\mu$g/ml. The specific activity of CMW-W was 3-fold greater than that of the initial methanol extracts and the yield of the activity exceeded 200%, suggesting that an interfering substance had been removed.

The CMW-W fraction was subjected to a subsequent HPLC purification step in which a preliminary HPLC elution pattern...
Blueberry leaves (Freeze-dried powder; 1.0 g) → Extraction with methanol → Methanol extracts (440 mg) [IC50: 5.47] → Addition of chloroform → Precipitate (CMW-ppt : 63.7 mg) → Addition of water and methanol → Chloroform extraction twice → Chloroform layer (CMW-C : 56.3 mg) [IC50: > 3.0] → Water layer (CMW-W : 284.2 mg) [IC50: 1.74] → HPLC 1st fractionation (LC1 : 140.2 mg) → HPLC 2nd fractionation (LC2 : 24.6 mg) → HPLC 3rd fractionation (LC3 : 2.9 mg) [IC50: 0.087]

**FIGURE 1. Fractionation of blueberry leaf extract for the inhibitor of HCV subgenome expression.** The inhibitory activity was indicated under each fraction as the IC50 value (micrograms/ml).

**TABLE 1**

| Purification of HCV subgenome expression inhibitory activity in blueberry leaf | Total weight | Subgenome expression, IC50 | Specific activity | Purification factor | Total activity | Yield |
|---|---|---|---|---|---|---|
| mg | µg/ml | 1/IC50 | mg/IC50 | % |
| MeOH extract | 440.0 | 5.47 | 0.18 | 1.00 | 80.44 | 100 |
| Water layer | 284.2 | 1.74 | 0.57 | 3.14 | 163.33 | 203.05 |
| LC1st | 140.2 | 0.89 | 1.12 | 6.15 | 157.53 | 195.84 |
| LC2nd | 24.6 | 0.54 | 1.85 | 10.13 | 45.56 | 56.63 |
| LC3rd | 2.9 | 0.087 | 11.49 | 62.87 | 33.33 | 41.44 |

(a 15–100% gradient of acetonitrile) was used. The data indicated that a strong inhibitory activity eluted around 90% of acetonitrile (17 min) with some minor inhibitory activities broadly eluted earlier. Those results suggested the possible existence of multiple HCV subgenome expression inhibitors in the CMW-W fraction (Fig. 2A). To purify the most active component, we initially separated the CMW-W isocratic at 30% acetonitrile and collected the active fraction eluted at 3.3–5.2 min (Fig. 2B). After repeated collection, we obtained 140.2 mg of active fraction (LC1) from 440 mg of methanol extracts. The IC50 value of this fraction for HCV RNA expression was 0.89 µg/ml, yielding a specific activity 6-fold higher than that of the initial methanol extracts (Table 1). In the second round HPLC (Fig. 2C), we fractioned LC1 as follows: 20% acetonitrile from 0 to 7.5 min, followed by 20–100% linear gradient of acetonitrile from 7.5 to 12.5 min. A highly active fraction was eluted from 11.9 to 13.2 min and collected (LC2), yielding 24.6 mg with an IC50 value 0.54 µg/ml (Table 1). In the third HPLC step (Fig. 2D), we applied LC2 and eluted with 40 – 65% methanol instead of acetonitrile. The active fraction was eluted from 3.2 to 6.2 min and collected (LC3), finally yielding 2.9 mg of solid material with a dark flesh color. The IC50 value for HCV RNA expression of LC3 was 0.087 µg/ml, with a 63-fold increase in specific activity relative to the initial methanol extracts (Table 1). We also checked the cytotoxic effect on replicon cells. The CC50 value of the cytotoxicity of LC3 was 18.5 µg/ml, and the selective index, which was calculated by dividing CC50 by IC50, was 212.6, showing a 16.5-fold higher selective index value compared with initial methanol extracts (Fig. 3).

**The Inhibitor of HCV Subgenome Expression Is Proanthocyanidin**—To analyze the constituent elements in the purified fraction LC3, EPMA was performed. This analysis indicated that the fraction is composed of carbon and oxygen, but not nitrogen (data not shown). In addition, trace amounts of calcium, sodium, potassium, and aluminum, which appeared to be contaminating elements, were also identified. Next, LC3 was analyzed by LC/MS-IT-TOF. Preliminary trials showed that analysis required the use of an APCI probe at 450 °C, and no signal was obtained at 250 °C. The mass spectrum data showed five peaks (Fig. 4), and [M-H]− at m/z 401.0494 and 689.1135 were considered to be trifluoroacetic acid adducts of m/z 287.0553 and 575.1196, respectively. From these spectra, the mass of this compound appeared to be [M-H]− at m/z 575.1196, which was estimated to be C28H22O12 (error = 0.17 ppm), an A-type dimer of procyanidin. Given the fact that strict conditions (APCI probe temperature at 450 °C) were required to ionize the compound, it appeared that the isolate consisted of one or more polymers of procyanidin.

We next analyzed the purified LC3 fraction by butanol-HCl hydrolysis (Porter method) (11, 12). The reacted solution turned a red color, which is in accordance with the color of anthocyanidin generated by heating of procyanidin/proanthocyanidin under acidic condition. Using procyanidin B2 as a standard, the procyanidin content in the LC3 fraction was 86.33%. The hydrolysis solution was analyzed by LC/MS-IT-TOF. The main peak (retention time = 7.3 min) of the PDA chromatogram at 540 nm was observed at the same position as that of the cyanidin standard (Fig. 5A). Indeed, MS/MS spectra of this peak were identical to those of the cyanidin standard (Fig. 5B). These results revealed that the HCV RNA replication inhibitory compound present in the LC3 fraction from blueberry leaves was procyanidin. Because the hydrolysate of this compound also contained a trace amount of delphinidin (Fig. 5A, arrow), this compound was considered to be proanthocyanidin rather than procyanidin.

**Structural Analysis of the Inhibitory Proanthocyanidin by Thiolysis**—To analyze the terminal and extension units and also define mDP of proanthocyanidin in the purified LC3 fraction of blueberry leaves, we combined thiolysis (13) with reversed-phase HPLC. When thiolysis products of purified proanthocyanidin in the LC3 fraction were analyzed in reversed-phase HPLC, several peaks (A–H) were identified (Fig. 6). The peaks A, C, and H were considered to be catechin, epicatechin, and benzylmercapatan, respectively, according to the retention time of each standard preparation. Other peaks were confirmed by analyzing mass spectra. The parent mass of peak E was [M-H]−
at m/z 411.0892, with an estimated formula of C_{22}H_{20}O_{6}S (error = −3.8 ppm), and its MS/MS spectrum was [M-H]− at m/z 287.0510. The difference between the parental mass and MS/MS was 124.0382, which was in accordance with a benzylthio adduct. Thus, peak E appeared to be catechin or epicatechin benzylthioether. Because the retention time of epicatechin benzylthioether was the same as that of peak E, we considered peak E to be epicatechin benzylthioether. The parental mass of peak G was [M-H]− at m/z 697.1385 (predicted formula: C_{37}H_{30}O_{12}S), and its MS/MS was [M-H]− at m/z 573.0987. Again, the difference was 124.0398 and likely represented the benzylthio adduct. Thus, peak G was estimated to be a benzylthioether of A-type dimer consisting of catechin and/or epicatechin. Peak B was detected as parent MS [M-H]− at m/z 863.1822 with a predicted formula C_{45}H_{36}O_{18} (error = −0.86 ppm). Because the formula of B-type procyanidin trimer is C_{45}H_{38}O_{18} and that of A-type is C_{45}H_{36}O_{18}, this peak was likely a trimer in which A-type and B-type interflavan bonds coexisted. Peak D was suggested to be an A-B type trimer similar to peak B but with a benzylthio adduct. The parental mass of peak F was [M-H]− at m/z 605.1449, and its MS/MS was [M-H]− at m/z 481.1109, so that a benzylthio adduct was also present in peak F. However, we could not obtain the predicted formula of the parental mass of peak F. The structural analysis of the HCV inhibitor proanthocyanidin from blueberry leaves (fraction LC3) is summarized in Table 2. The mDP of proanthocyanidin in this fraction was estimated to be 7.7. Because the predicted formula of peak F was undefined, peak F is indicated as “unknown” in Table 2.

Role of Polymerized Structure of Proanthocyanidin in the Inhibition of HCV Subgenome Expression—Because the purified HCV expression-inhibitory proanthocyanidin of blueberry leaf was oligomer with mDP 7.7, we asked whether the polymerization was required for inhibitory activity. First, the inhibitory activities of monomers such as catechin, epicatechin, and epigallocatechin-gallate, all of which were constituents of proanthocyanidin, and also of the dimer (procyanidin B2) were
Blueberry Leaf Proanthocyanidin Suppresses HCV

We then determined how the degree of polymerization of proanthocyanidin affected the inhibition. The crude fraction of proanthocyanidins was obtained by the extraction of three low polarity solvents (acetone-hexane-ethyl acetate) as described under “Experimental Procedures.” The IC$_{50}$ of HCV RNA expression of this proanthocyanidin-enriched fraction was 3.20 μg/ml, showing greater activity than the crude methanol extract. After fractionation on a Sephadex LH-20 column, each eluant was analyzed by LC/MS-IT-TOF and thiolysis to determine the components and mDP of proanthocyanidin (supplemental Fig. S1). Then, the blueberry leaf-derived proanthocyanidins with different mDP were assessed for HCV inhibitory activity. The inhibitory activity of blueberry leaf proanthocyanidin was clearly dependent on the polymerization level, and the peak activity was observed at a polymerization level of $\sim$8 to 9 (IC$_{50}$: 0.05 μg/ml) (Fig. 7).

Effect of Purified Blueberry Proanthocyanidin on the Expression of NS3 HCV Protein in Replicon Cells—In our system, HCV RNA expression in replicon cells was expressed as luciferase activity. Thus, the observed inhibitory activity may have resulted from nonspecific inhibition of luciferase by proanthocyanidin. Therefore, we examined the effect of the purified proanthocyanidin (fraction LC3) on the expression levels of the neomycin-resistant gene and the NS3 protein gene, both of which were encoded in the HCV subgenome of replicon cells. The purified blueberry proanthocyanidin suppressed the expression of the neomycin-resistant gene and also the levels of NS3 protein in a concentration-dependent manner, indicating that the proanthocyanidin purified from blueberry leaves in fact suppressed the expression of HCV subgenome in the replicon cells (Fig. 8).

hnRNP A2/B1, Which Has Affinity to Proanthocyanidin, Is Indispensable for Expression of Subgenomic HCV RNA—To investigate the molecular mechanism underlying the suppression of HCV RNA expression by proanthocyanidin, we comprehensively identified proteins having affinity to the purified proanthocyanidin from blueberry leaves. The protein extract from replicon cells was treated with proanthocyanidin-coupled Sepharose, and then the adsorbed proteins were eluted. The extract was also treated with Sepharose beads coupled to catechin, a structural unit of proanthocyanidin, but HCV subgenome-expression inhibitory activity was not observed (Table 3). The proteins having higher affinity to proanthocyanidin than catechin were detected with fluorescent two-dimensional-DIGE (Fig. 9). In the eluate from proanthocyanidin-coupled Sepharose, intensities of 32 spots were increased compared with those from catechin-coupled Sepharose. Twenty-seven spots were cut from Coomassie-stained gels and subjected to peptide mass fingerprinting using MS, and we successfully identified proteins derived from 25 spots (Nos. 1 to 25 in Fig. 9A and Table 4). Although other possible candidate spots were also suggested in a rectangular portion (Fig. 9A), they were not subjected to protein identification due to insufficient separation.

From the list of identified proteins (Table 4), most could be categorized into two groups. The first group consisted of subunits of eukaryotic translation initiation factor 3 (eIF3). They included eIF3A (spot Nos. 1, 5, and 9), eIF3F (No. 10), eIF3G (No. 12), eIF3H (No. 4), and eIF3M (No. 13). Although eIF3A was identified from multiple protein spots (Nos. 1, 5, and 9), this may be due to post-translational modification and protein processing. The second group of proteins consisted of hnRNPs such as hnRNP A/B (No. 19), hnRNP A2/B1.
hnRNP K (Nos. 17 and 22), hnRNP L (Nos. 11, 15, and 21), and hnRNP Q (Nos. 2, 6, and 7) also known as NS1-associated protein 1. Importantly, eIF3 has been reported to bind directly to the HCV internal ribosome entry site (IRES), leading to translation initiation of viral proteins (18). Moreover, all hnRNPs identified have been reported to be associated with HCV genomic RNA such as IRES and non-translated regions (19–25). These results imply that proanthocyanidin may target cellular proteins such as eIF3 and hnRNPs. To further clarify the relationship between these proteins and HCV subgenomic expression, we examined the effects of siRNA-based knockdown of these proteins (supplemental Fig. S2). First, we selected three eIF3 subunits (eIF3F, eIF3G, and eIF3H), which are thought to be involved in IRES binding of eIF3 (26). However, knockdown of these subunits did not affect the luciferase activity in replicon cells. Then, we targeted all hnRNPs identified. Among them, siRNA pool targeting hnRNP A2/B1 significantly suppressed the luciferase activity of HCV subgenomic replicon cells (supplemental Fig. S2), and this result was confirmed using two kinds of single siRNA (Fig. 10). Weak suppressive activities were also suggested by siRNAs targeting other hnRNPs such as hnRNP A/B, K, and L (supplemental Fig. S2).

DISCUSSION

The HCV infection is a major cause of chronic liver disease, which eventually results in end-stage liver diseases such as cirrhosis and hepatocellular carcinoma. A crude extract from rabbit-eye blueberry (V. virgatum Aiton) leaves exhibited significant inhibitory activity against HCV RNA expression when analyzed in HCV subgenomic replicon cells. In this study, we attempted to purify a compound that suppresses HCV subgenome expression from the blueberry leaves. The final purified product was identified as proanthocyanidin, and it was effective at concentrations that are two orders of magnitude below the toxic threshold in replicon cells. The mDP of the proanthocyanidin in purified anti-HCV expression fraction was 7.7 with a high proportion of epicatechin as the monomeric components. Subsequent analysis indicated that the blueberry leaf-derived proanthocyanidin with a degree of polymerization of ~8–9 shows the highest inhibitory activity. Finally, the purified pro-
anthocyanidin from blueberry leaf extracts suppressed the expression of the neomycin phosphotransferase gene and the NS-3 protein gene in HCV subgenome replicon cells in a dose-dependent manner. These data suggest the potential value of blueberry leaf proanthocyanidin for the treatment of HCV infection.

Proanthocyanidin is a polyphenol that shows polymerization of more than two units of flavan-3-ol such as catechin and epicatechin (supplementalFig. S3). There are two interflavan bonds in proanthocyanidin, in which the B-type has one linkage of interflavan bond (C4→C8 or C4→C6) and the A-type has two linkages of bonds (C4→C8 and O7→C2) (27). Proanthocyanidins were previously known as condensed tannin and are present in various plants and foods. They contribute to organoleptic properties such as stability, astringency, and bitterness (28, 29). There are a number of foods and nutritional supplements that contain proanthocyanidins with health-promoting benefits, and their value has been described in the literature and patent documents. For example, proanthocyanidin contained in blueberries increases the lifespan of the nematode (Caenorhabditis elegans) (30).

**Blueberry Leaf Proanthocyanidin Suppresses HCV**

![Figure 7: Scatter plot of mDP and specific activity of subgenomic HCV RNA-expression inhibition](Image)

**TABLE 2**

| Compounds                  | DP or mDP | Subgenomic expression, IC50 | Cytotoxicity, CC50 | Ratio, CC50/IC50 |
|----------------------------|-----------|----------------------------|--------------------|-----------------|
| Catechin                   | 1         | 16.18                      | 100.4              | 6.2             |
| Epicatechin                | 1         | 27.32                      | 113.8              | 4.2             |
| Epigallocatechin-gallate   | 1         | 14.61                      | 41.68              | 2.9             |
| Procyanidin B2a            | 2         | >25.0                      | >25.0              | --              |
| Purified proanthocyanidin from blueberry leaf (LC3 fraction) | 7.7 | 0.087 | 18.5 | 212.0 |

* Epicatechin dimer.

**TABLE 3**

| Compounds                  | IC50 g/ml proanthocyanidin in a 3-fold dilution series. The expression of β-actin mRNA is also indicated as a normalization control. | CC50 g/ml proanthocyanidin in a 3-fold dilution series. The β-actin protein levels are also shown as a normalization control. |
|----------------------------|---------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| Neo                       | A Northern blot analysis of the neomycin-resistant gene expression (Neo) in the presence of 0 µg/ml (control) to 3.3 µg/ml proanthocyanidin in a 3-fold dilution series. The expression of β-actin mRNA is also indicated as a normalization control. | A Western blot analysis of the expression of NS-3 protein (NS3) in the presence of 0 µg/ml (control) to 10 µg/ml proanthocyanidin in a 3-fold dilution series. The β-actin protein levels are also shown as a normalization control. |
| Actin                     | A Northern blot analysis of the neomycin-resistant gene expression (Neo) in the presence of 0 µg/ml (control) to 3.3 µg/ml proanthocyanidin in a 3-fold dilution series. The expression of β-actin mRNA is also indicated as a normalization control. | A Western blot analysis of the expression of NS-3 protein (NS3) in the presence of 0 µg/ml (control) to 10 µg/ml proanthocyanidin in a 3-fold dilution series. The β-actin protein levels are also shown as a normalization control. |

* Epicatechin dimer.

**FIGURE 7. Scatter plot of mDP and specific activity of subgenomic HCV RNA-expression inhibition**. The mDP was estimated by thiolysis of each fraction. The specific activity was calculated from IC50 value of each fraction.
inhibitors of IRES-directed translation in HCV-infected cells, vitamin B12, a synthetic peptide derived from human La protein, and RNA molecules targeting IRES have been reported (42–44). However, little is known regarding the effect of natural product-derived polyphenolic compounds on HCV IRES-directed translation, and this possibility should be clarified in a future study. It should be noted that all proanthocyanidin-binding proteins identified in this study are intracytoplasmic and/or intranuclear proteins. However, it is not known whether proanthocyanidin can be efficiently translocated into the intracellular space despite its highly polymerized structure. Nonetheless, absorption of proanthocyanidin from the digestive tract has been reported (12, 45), suggesting the possibility of proanthocyanidin internalization into cells, and internalization of high molecular weight molecule via clathrin-mediated endocytosis, caveolae-mediated uptake or pinocytosis has been reported (46). Further studies are in progress, focusing on the intracellular uptake of proanthocyanidin.

The current therapies for hepatitis C patients are based on a combination of pegylated recombinant interferons and ribavirin. However, viral clearance is achieved by <60% of treated patients, and the therapies are limited by significant side effects and high costs (47, 48). Therefore, many novel anti-HCV drugs are currently under development, most of which target viral enzymes. For example, BILN-2061, VX-950, and SCH503034 are inhib-

**TABLE 4**

| Spot no. | Intensity a (%) (×10^6) | Ratio b | p value c | Protein name d | Accession number e | Coverage g | Molecular mass h (kDa) | pI i |
|----------|-------------------------|---------|------------|----------------|--------------------|------------|------------------------|------|
| 1        | 5.69 ± 2.98             | 0.99 ± 0.24 | 5.73       | Leucine-rich PPR motif-containing protein, mitochondrial | Q42704    | 10.3                  | 159.0  | 5.81                  |
| 2        | 6.19 ± 1.33             | 1.39 ± 0.30 | 5.00       | hnRNPL         | Q41452    | 12.4                  | 166.9 | 6.38                  |
| 3        | 5.63 ± 0.71             | 1.15 ± 0.36 | 4.88       | Splicing factor U2AF 65-kDa subunit | P2628      | 22.5                  | 53.8  | 9.19                  |
| 4        | 8.19 ± 2.57             | 1.68 ± 0.35 | 4.86       | eIF3H          | Q15372    | 41.2                  | 40.1  | 6.09                  |
| 5        | 5.22 ± 2.84             | 1.33 ± 0.36 | 3.91       | eIF3A          | Q14412    | 15.1                  | 166.9 | 6.38                  |
| 6        | 6.02 ± 1.76             | 2.28 ± 0.90 | 3.52       | hnRNPL         | O60506    | 24.4                  | 69.8  | 8.68                  |
| 7        | 2.45 ± 0.29             | 0.73 ± 0.24 | 3.35       | hnRNPL         | O60506    | 16.4                  | 69.8  | 8.68                  |
| 8        | 17.11 ± 3.99            | 5.24 ± 4.26 | 3.26       | hnRNPL A2/B1   | P22626    | 36.3                  | 37.5  | 8.97                  |
| 9        | 2.66 ± 1.70             | 1.00 ± 0.29 | 2.65       | eIF3A          | Q14412    | 15.2                  | 166.9 | 6.38                  |
| 10       | 2.37 ± 0.82             | 0.96 ± 0.19 | 2.47       | eIF3F          | O00303    | 28.0                  | 37.7  | 5.24                  |
| 11       | 5.40 ± 1.55             | 2.27 ± 0.56 | 2.38       | hnRNPL         | P14866    | 20.2                  | 64.7  | 8.46                  |
| 12       | 6.77 ± 3.52             | 2.86 ± 0.68 | 2.37       | eIF3G          | O75821    | 16.3                  | 35.9  | 5.87                  |
| 13       | 17.99 ± 9.05            | 7.89 ± 2.54 | 2.28       | eIF3M          | Q71247    | 32.6                  | 42.9  | 5.41                  |
| 14       | 9.28 ± 1.10             | 4.26 ± 0.71 | 2.18       | <0.0001        | hnRNPL L  | 9.05                  | 7.89  | 6.38                  |
| 15       | 6.78 ± 1.96             | 3.20 ± 0.75 | 2.12       | Leucine-rich PPR motif-containing protein, mitochondrial | P42704    | 10.3                  | 159.0 | 5.81                  |
| 16       | 2.54 ± 0.55             | 1.24 ± 0.11 | 2.05       | Splicing factor U2AF 65-kDa subunit | P26368    | 20.0                  | 53.8  | 9.19                  |
| 17       | 17.65 ± 1.23            | 9.76 ± 1.87 | 1.81       | hnRNPL K       | P61978    | 31.3                  | 51.2  | 5.39                  |
| 18       | 32.71 ± 6.34            | 19.20 ± 6.08 | 1.70     | Splicing factor, proline- and glutamine-rich | P23246    | 19.4                  | 76.2  | 9.45                  |
| 19       | 3.98 ± 0.35             | 2.34 ± 0.32 | 1.70       | <0.0001        | Heterogeneous nuclear ribonucleoprotein A/B (hnRNPL A/B) | Q99729    | 17.5                  | 36.3  | 8.22                  |
| 20       | 3.57 ± 0.59             | 2.13 ± 0.52 | 1.68       | <0.0001        | Splicing factor 45 | Q96425    | 17.0                  | 45.2  | 5.76                  |
| 21       | 4.22 ± 1.18             | 2.52 ± 0.32 | 1.68       | hnRNPL        | P14866    | 21.2                  | 64.7  | 8.46                  |
| 22       | 28.44 ± 2.99            | 17.40 ± 3.82 | 1.63     | hnRNPL K       | P61978    | 29.8                  | 51.2  | 5.39                  |
| 23       | 18.62 ± 1.68            | 11.76 ± 3.01 | 1.58     | ATP-dependent RNA helicase DDX1 | Q92499    | 40.3                  | 83.3  | 6.81                  |
| 24       | 1.81 ± 0.46             | 1.18 ± 0.35 | 1.53       | Fragile X mental retardation syndrome-related protein 1 | P51114    | 15.5                  | 70.0  | 5.84                  |
| 25       | 5.42 ± 0.57             | 3.60 ± 0.93 | 1.51       | Splicing factor U2AF 65-kDa subunit | P26368    | 26.3                  | 53.8  | 9.19                  |

a Spot numbers correspond to those in Fig. 9.

b Intensities of spots are shown as normalized volume ± S.D. (nine gels per group; proanthocyanidin and catechin).

c Ratio was calculated using Progenesis Discovery software and expressed as differences of spot intensity in proteins eluted from proanthocyanidin-coupled Sepharose compared with those from catechin-coupled Sepharose.

d Statistical difference were determined by Student’s t test. Values of p < 0.05 were considered significant.

e Proteins were identified using Mascot with Swiss-Prot database.

f References for identified proteins.

g Percentage cover of the identified peptide in total tryptic digests.

h Theoretical molecular mass from Mascot search results.

i Theoretical isoelectric point (pI) from Mascot search results.
Blueberry Leaf Proanthocyanidin Suppresses HCV

The Flavonoids

Acknowledgments—We thank Kazunori Nakata (Miyazaki Prefecture Industrial Technology Research Center) for support in the EPMA analysis and Dr. Michinori Kohara (The Tokyo Metropolitan Institute of Medical Science) for providing HCV replicon cells.

REFERENCES

1. Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Science 244, 359–362
2. Seeff, L. B. (1999) Am. J. Med. 107, 105–155
3. Di Biscaglia, A. M., Order, S. E., Sjogren, M. H., Kuo, G., Houghton, M., and Choo, Q. L. (1991) Am. J. Gastroenterol. 86, 335–338
4. Myles, D. C. (2001) Curr. Opin. Drug Discov. Devel. 4, 411–416
5. Dillon, J. F. (2004) J. Viral. Hepat. 11, Suppl. 1, 23–27
6. Ni, Z. J., and Wagner, A. S. (2004) Curr. Opin. Drug Discov. Devel. 7, 446–459
7. Manns, M. P., McHutchison, J. G., Gordon, S. C., Rustgi, V. K., Shiffman, M., Reindollar, R., Goodman, Z. D., Koury, K., Ling, M., and Albrecht, J. K. (2001) Lancet 358, 956–965
8. Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marininos, G., Gonçalves, F. L., Jr., Häussinger, D., Diago, M., Carosi, G., Dhumé, D., Craxi, A., Lin, A., Hoffman, J., and Yu, J. (2002) N. Engl. J. Med. 347, 975–982
9. De Franceschi, L., Fattovich, G., Turrfini, F., Ayi, K., Brugnara, C., Manzato, F., Noventa, F., Stanzial, A. M., Solero, P., and Corrocher, R. (2000) Hepatology 31, 997–1004
10. Sakamoto, H., Okamoto, K., Aoki, M., Kato, H., Katsume, A., Ohta, A., Tsukuda, T., Shimma, N, Aoki, Y., Arisawa, M., Kohara, M., and Sudoh, M. (2005) Nat. Chem. Biol. 1, 333–337
11. Porter, L. J., Hrstimich, L. N., and Chan, B. G. (1986) Phytochemistry 25, 223–230
12. Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., Kanda, T., Ohatake, Y., and Goda, Y. (2006) J. Agric. Food Chem. 54, 894–892
13. Guyot, S., Marnet, N., and Driluele, J. F. (2001) J. Agric. Food Chem. 49, 14–20
14. Gu, L., Kelm, M., Hammerstone, J. F., Beecher, G., Cunningham, D., Van-nozzi, S., and Prior, R. L. (2002) J. Agric. Food Chem. 50, 4852–4860
15. Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, I., Pognan, F., Hawkins, E., Currie, I., and Davison, M. (2001) Proteomics 1, 377–396
16. Marouga, R., David, S., and Hawkins, E. (2005) Anal. Bioanal. Chem. 382, 669–678
17. Schvchenko, A., Jensen, O. N., Podtelejenkov, A. V., Sagliocco, F., Wilm, M., Orom, M., Mortensen, P., Schvchenko, A., Boucherie, H., and Mann, M. (1999) Proc. Natl. Acad. Sci. U.S.A. 93, 14440–14445
18. Siridechadilok, B., Fraser, C. S., Hall, R. J., Doudna, J. A., and Nogales, E. (2003) Science 310, 1513–1515
19. Hahn, B., Kim, Y. K., Kim, J. H., Kim, T. Y., and Jang, S. K. (1998) J. Virol. 72, 8782–8788
20. Lu, H., Li, W., Noble, W. S., Payan, D., and Anderson, D. C. (2004) J. Proteome Res. 3, 949–957
21. Kim, J. H., Paek, K. Y., Ha, S. H., Cho, S., Choi, K., Kim, C. S., Ryu, S. H., and Jang, S. K. (2004) Mol. Cell. Biol. 24, 7878–7890
22. Harris, D., Zhang, Z., Chaubey, B., and Pandey, V. N. (2006) Mol. Cell. Proteomics 5, 1006–1018
23. Kim, C. S., Seol, S. K., Song, O. K., Park, J. H., and Jang, S. K. (2007) J. Virol. 81, 3852–3865
24. Pacheco, A., Reigadas, S., and Martínez-Salas, E. (2008) Proteomics 8, 4782–4790
25. Zhou, M., Sandercock, A. M., Fraser, C. S., Ridlova, G., Stephens, E., Schenauer, M. R., Yokoi-Fong, T., Barsky, D., Leary, J. A., Hershew, J. W., Doudna, J. A., and Robinson, C. V. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 18139–18144
26. Porter, L. J. (1988) In The Flavonoids (Harbone, J. B., ed) pp. 21–63, Chapman and Hall Ltd., New York
27. Arnold, R. A., and Noble, A. C. (1978) Am. J. Enol. Vitic. 29, 150–152
28. Brossaud, F., Cheynier, V., and Noble, A. C. (2001) Aust. J. Grape Wine Res. 7, 33–39
29. Wilson, M. A., Shukitt-Hale, B., Kalt, W., Ingram, D. K., Joseph, J. A., and Wolkow, C. A. (2006) Aging Cell 5, 59–68
30. Chen, Z. P., Cai, Y., and Phillipson, J. D. (1994) Planta Med. 60, 541–545

FIGURE 10. Effects of hnRNP A2/B1 knockdown on HCV subgenome expression in replicon cells. Results of two siRNAs sequences (si-#09 and si-#11) are shown. A, effects of siRNA on the expression of hnRNP A2/B1 protein. Same blot was also probed by anti-actin antibody. B, effects of siRNA on luciferase activity (HCV subgenome-expression activity) (closed bars) and cellular viability (open bars). The siRNA concentration is indicated as a logarithmic scale. Values are mean ± S.D. of triplicate experiments. *, p < 0.05; **, p < 0.001, Student t test.

inhibitors of NS3/4A serine protease, and R1479 and HCV-796 are inhibitors of NSSB RNA-dependent RNA polymerase (41, 48–53). NA255 is also an HCV replication inhibitor targeting the host sphingolipid biosynthesis (10). These compounds are relatively low in molecular weight and can be manufactured by organic synthesis. On the other hand, the anti-HCV compound purified from blueberry leaves is a flavan-3-ol polymer with a molecular mass of ~2 kDa. The highly polymerized structure that is required for the efficient inhibition of HCV subgenome expression makes synthesizing the anti-HCV proanthocyanidin difficult. However, because proanthocyanidins are components of many plants and foods, daily intake of proanthocyanidin is possible and may be beneficial against HCV replication in hepatitis C patients. We estimate that fresh blueberry leaf is rich in proanthocyanidin, which accounts for 3–4% of the weight. Moreover, the polymerized status of blueberry leaf-derived proanthocyanidin appears to be suitable for the inhibition of HCV subgenome expression. Therefore, blueberry leaves might have potential as a source of anti-HCV proanthocyanidin.

In summary, we demonstrated that extracts of blueberry leaf possess strong suppressive effects against HCV subgenome expression in a replicon cell system. We identified the inhibitor as a proanthocyanidin oligomer with an mDP value of ~8. Further studies of the mechanism underlying proanthocyanidin-mediated HCV inhibition may open new ways to design novel anti-HCV drugs.
Blueberry Leaf Proanthocyanidin Suppresses HCV

32. Williams, J. E. (2001) Altern. Med. Rev. 6, 567–579
33. Sidwell, R. W., Huffman, J. H., Moscon, B. J., and Warren, R. P. (1994) Chemotherapy 40, 42–50
34. Gilbert, B. E., Wyde, P. R., Wilson, S. Z., and Meyerson, L. R. (1993) Antiviral Res. 21, 37–45
35. Orozco-Topete, R., Sierra-Madero, J., Cano-Dominguez, C., Kershensovich, J., Ortiz-Pedroza, G., Vazquez-Valls, E., Garcia-Cosio, C., Soria-Cordoba, A., Armendariz, A. M., Teran-Toledo, X., Romo-Garcia, J., Fernandez, H., and Rozhon, E. J. (1997) Antiviral Res. 35, 91–103
36. Safrin, S., McKinley, G., McKeough, M., Robinson, D., and Spruance, S. L. (1994) Antiviral Res. 25, 185–192
37. Barnard, D. L., Smee, D. F., Huffman, J. H., Meyerson, L. R., and Sidwell, R. W. (1993) Chemotherapy 39, 203–211
38. Barnard, D. L., Huffman, J. H., Meyerson, L. R., and Sidwell, R. W. (1993) Chemotherapy 39, 212–217
39. Cheng, H. Y., Lin, C. C., and Lin, T. C. (2002) Antivir. Chem. Chemother. 13, 223–229
40. Nahmias, Y., Goldwasser, J., Casali, M., van Poll, D., Wakita, T., Chung, R. T., and Yarmush, M. L. (2008) Hepatology 47, 1437–1445
41. McCown, M. F., Rajyaguru, S., Le Pogam, S., Ali, S., Jiang, W. R., Kang, H., Symons, I., Cammack, N., and Najera, I. (2008) Antimicrob. Agents Chemother. 52, 1604–1612
42. Li, D., Lott, W. B., Martyn, J., Haqshenas, G., and Gowans, E. J. (2004) J. Virol. 78, 12075–12081
43. Pudi, R., Ramamurthy, S. S., and Das, S. (2005) J. Virol. 79, 9842–9853
44. Romero-López, C., Díaz-González, R., and Berzal-Herranz, A. (2007) Cell. Mol. Life Sci. 64, 2994–3006
45. Xu, L., Li, B., Cheng, M., Zhang, W., Pan, J., Zhang, C., and Gao, H. (2008) Exp. Clin. Endocrinol. Diabetes 116, 215–224
46. Kovtun, Y. V., and Goldmacher, V. S. (2007) Cancer Lett. 255, 232–240
47. Manns, M. P., Wedemeyer, H., and Cornberg, M. (2006) Gut 55, 1350–1359
48. Pawlotsky, J. M., and Gish, R. G. (2006) Antivir. Ther. 11, 397–408
49. Stuber, R. E., and Stadlbauer, V. (2006) J. Clin. Virol. 36, 87–94
50. Marlarre, D., Anderson, P. C., Bailey, M., Beaulieu, P., Bolger, G., Bonnaud, P., Bös, M., Cameron, D. R., Cartier, M., Cordingly, M. G., Faucher, A. M., Goudreau, N., Kawai, S. H., Kukolj, G., Lagacé, L., LaPlante, S. R., Narjes, H., Poupard, M. A., Rancourt, J., Sentjens, R. E., St George, R., Simoneau, B., Steinmann, G., Thibeault, D., Tsantrizos, Y. S., Weldon, S. M., Yong, C. L., and Llinás-Brunet, M. (2003) Nature 426, 186–189
51. Reesink, H. W., Zeuzem, S., Weegink, C. J., Forestier, N., van Vliet, A., van de Wetering de Rooij, J., McNair, L., Purdy, S., Kauffman, R., Alam, J., and Jansen, P. L. (2006) Gastroenterology 131, 997–1002
52. Sarrazin, C., Kieffer, T. L., Bartels, D., Hanzelka, B., Mühl, U., Welker, M., Wincheringer, D., Zhou, Y., Chu, H. M., Lin, C., Weegink, C., Reesink, H., Zeuzem, S., and Kwong, A. D. (2007) Gastroenterology 132, 1767–1777
53. Sarrazin, C., Rouzier, R., Wagner, F., Forestier, N., Larrey, D., Gupta, S. K., Hussain, M., Shah, A., Cutler, D., Zhang, J., and Zeuzem, S. (2007) Gastroenterology 132, 1270–1278