Anti-hepatitis C Virus Effect of Citrus Unshiu Peel and Its Active Ingredient Nobiletin

Megumi Suzuki,* Kenroh Sasaki,† Fumihiko Yoshizaki,† Min Fujisawa* Katsuji Oguchi* and Jong-Chol Cyong‡

*Department of Pharmacology, School of Medicine, Showa University, Japan
†Tohoku Pharmaceutical University, Japan
‡Nihon Pharmaceutical University, Japan

Abstract: We investigated the effects of water and ethyl acetate extracts of Citrus unshiu peel (Aurantii Nobilis pericarpium) on hepatitis C virus (HCV) absorption in MOLT-4 cells (a human lymphoblastoid leukemia cell line). By reverse transcription polymerase chain reaction (RT-PCR), we showed that both the ethyl acetate layer of Citrus unshiu peel extract and fraction 7 decreased HCV absorption in MOLT-4 cells. Furthermore, we demonstrated that 3',4',5,6,7,8-hexamethoxyflavone (nobiletin) is the active ingredient that markedly inhibited HCV infection in MOLT-4 cells.

Keywords: Hepatitis C Virus (HCV); Ninjin-Yoei-To (Formula Ginseng Composite: TJ-108); Citrus Unshiu Peel (Aurantii Nobilis Pericarpium); 3',4',5,6,7,8-Hexamethoxyflavone (Nobiletin).

Introduction

In 1989, the hepatitis C virus (HCV) was first identified as the etiologic agent of post-transfusion non-A and non-B hepatitis. Interferon (IFN) is regarded as the effective treatment against HCV in the majority of cases; however, it has been reported that one-third of the cases do not respond to this treatment. Furthermore, little effect of IFN therapy can be expected for most Japanese patients infected with HCV type 1b (Yoshioka et al., 1992). HCV1b viral titers in blood of these Japanese patients are higher than those of other genotype HCV. Moreover, IFN is contraindicated for use in elderly patients or those with autoimmune disease, mental disorders, cirrhosis, diabetes, and hypothyroidism or hyperthyroidism. In these patients however, Kampo herbal medicine seems clinically effective.

Correspondence to: Dr. Jong-Chol Cyong, Nihon Pharmaceutical University, 3-15-7-8F Nihonbashi Chou-Ku, Tokyo 103-0027, Japan. Tel/Fax: (+81) 3-3272-6966, E-mail: cyong@lapis.plala.or.jp
Various kinds of Kampo herbal medicines have been used to treat HCV-infected patients for many years in our institution. Previously we have reported that Ninjin-yoei-to (Formula ginseng compositae) eradicates HCV in the serum of patients with chronic hepatitis C (Cyong and Furuya, 1995). Ninjin-yoei-to is a mixture of raw herbs including Rehmannia root (Rehmanniae Radix), Japanese angelica root (Angelicae Radix), Atractylodes rhizome (Atractylodis Rhizoma), Hoelen (Hoelen), Ginseng root (Ginseng Radix), Cinnamon bark (Cinnamomi Cortex), Polygalal root (Polygalae Radix), Peony root (Paeoniae Radix), Citrus unshiu peel (Aurantii Nobilis pericarpium), Astragalus root (Astragali Radix), Glycyrrhiza root (Glycyrrhizae Radix), and Schisandra fruit (Schisandraceae Fructus) (Cyong et al., 2000). The MOLT-4 cell line of human T-cell origin is the only known cell line that may be infected by HCV (Shimizu et al., 1992). We have demonstrated that, among the herbal ingredients of Ninjin-yoei-to, Schisandra fruit and Citrus unshiu peel inhibit HCV infection in MOLT-4 cells (Cyong et al., 2000). We also demonstrated that the active ingredient in Schisandra fruit is gomisin A (Cyong et al., 2000). The aim of the present study was hence to determine the active ingredients in Citrus unshiu peel that inhibit HCV infection in MOLT-4 cells.

Materials and Methods

Anti-HCV Effect of Serum Treated with Citrus Unshiu Peel

Citrus unshiu peel water extract was administered to a healthy volunteer subject once/day for 3 days. On the 3rd day, blood samples were collected 2 hours after administration of Citrus unshiu peel. Serum was isolated and heat-inactivated. Serum treated with Citrus unshiu peel (10%) was added to MOLT-4 cell culture medium (see below) instead of FBS. HCV-positive serum was also added to examine the inhibitory effects of Citrus unshiu peel on HCV infection.

Cell Line

Human MOLT-4 lymphoblastoma cells (MOLT-4 cells) were purchased from Dainippon Pharmaceutical Co. Ltd. (Japan). The cells were grown in 75 cm² culture flasks (Falcon, USA) in RPMI 1640 medium (GIBCO BRL, MD, USA) at 37°C in a humidified 5% CO₂ incubator. The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, KA, USA).

Preparation of Extracts

Citrus unshiu peel was purchased from Uchida Wakanryaku Co. (Tokyo, Japan). The voucher specimen number was TG352417. Citrus unshiu peel (15 g) was extracted with 300 ml distilled water to the half volume, and the supernatant then was centrifuged at 3500 rpm for
20 minutes. After aspirating filtration, the solution was adjusted to 150 ml. Polychlal AT (Gokyo sangyo, Japan) 1.5 g was added to the extract and the mixture was shaken for 30 minutes at room temperature to eliminate tannic acid. After elimination, the solution was filtered and adjusted to 150 ml. This solution was used as a water extract for the first screening. Next, material was extracted and fractionated according to the procedure of Sasaki and Yoshizaki (2002). The crude drug 3.5 kg was extracted twice with methanol (8 L) at room temperature. Removal of solvent \textit{in vacuo} gave 952.2 g of methanol extract. The extract was dissolved in water and distributed three times in chloroform (1 L) to give a chloroform extract (31.6 g). The water-soluble residue was partitioned three times between 1 L of ethyl acetate (EtOAc) and 1 L of H$_2$O. After removal of the solvent \textit{in vacuo}, the EtOAc extract (27.3 g) was applied to gradient column chromatography on silica gel (Wakogel C-200) using n-hexane/acetone (0/100–100/0) as eluents. The volumes of eluate were 200 ml each. The proportions of elutions and yields of extracts were as follow: fr.1, 100/0 for 5.55 g; fr.2, 96/4 for 0.42 g; fr.3, 90/10 for 0.64 g; fr.4, 80/20 for 1.35 g; fr.5, 70/30 for 1.73 g; fr.6, 60/40 for 2.59 g; fr.7, 60/40 for 2.26 g; fr.8, 50/50 for 1.15 g; fr.9, 40/60 for 1.06 g; fr.10, 30/70 for 1.31 g; fr.11, 20/80 for 1.39 g. Fr.7 furnished colorless needles (recrystallized from acetone/methanol), mp 139–140.5°C, yielding about 65 mg. This compound was identified as 3’,4’,5,6,7,8-hexamethoxyflavone (nobiletin) according to the spectral data as compared with those in the literature. All the extracts or fractions of Citrus unshiu peel were dissolved in ethanol and applied to the cell culture after dilution to 0.01–100 µg/ml with RPMI 1640 medium.

**HCV Positive Serum**

HCV type Ib-positive serum 2400 kIU/ml was collected from the peripheral blood of untreated patients. IFN-β treatment at doses of 0.1–100 µg/ml was ineffective at eradicating the virus in these blood samples.

**Assay for HCV**

The method of detecting HCV infection was based on that of Shimizu \textit{et al.} (1992). MOLT-4 cells (900 µl) were placed in 24-well plates (5 × 10$^5$ cells/well) and an appropriate quantity of extracts (100 µl) was also added to each well. Cells were cultured at 37°C with 5% CO$_2$ for 48 hours. Virus-positive serum 10 µl was then added to each well and the cells were incubated at 37°C for another 4 hours to facilitate absorption of the virus. After washing five times with PBS, RNA was extracted by RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions for the following nested reverse transcription polymerase chain reaction (RT-PCR). The amount of virus was subsequently measured by RT-PCR (Honda \textit{et al.}, 1994). Each assay was performed three times to check reproducibility.
Assay for Cytotoxicity

MOLT-4 cells were cultured as described previously. Cytotoxicity was measured using Alamar Blue (Biosource International Inc., CA, USA). Alamar Blue (10 µl) was added to each well and incubated at 37°C with 5% CO₂ for 4 hours, then absorbance was measured at 570 nm with a microplate reader (Bio-Rad model 550, USA) to assess the effect of each sample on viability of the cells.

Statistical Analysis

Data were analyzed by Student’s t-test. P < 0.05 were considered significant.

Results

Anti-HCV Effect

When human serum treated with Citrus unshiu peel was added to HCV-infected MOLT-4 cell culture medium, RT-PCR band was significantly reduced compared with the positive control (Fig. 1).

Effect of Citrus Unshiu Peel Extract

At first screening, water extract of Citrus unshiu peel inhibited HCV infection in MOLT-4 cells in a dose-dependent manner (Fig. 2). Extracts were further partitioned to chloroform, EtOAc, n-butanol and H₂O. Only the EtOAc layer inhibited HCV infection in MOLT-4 cells, at a dose of 100 µg/ml. The EtOAc layer was then divided by column chromatography into 11 fractions, each of which was examined for anti-HCV activity and cytotoxicity. Only fr.7 inhibited HCV infection at a dose of 10 µg/ml. This fraction yielded about 65 mg of crystal product, which was determined to be 3′,4′,5,6,7,8-hexamethoxyflavone (nobiletin) (Fig. 3) (Chen et al., 1997). Nobiletin inhibited HCV infection in MOLT-4 cells in a dose-dependent manner (Fig. 4A). The recovery rate of activity was 5.5% in the EtOAc layer and 4.6% in fr.7. Nobiletin treatment at concentrations ≥ 0.2 µg/ml had significant (P < 0.001) anti-HCV effects compared with the control (Fig. 4B).

Discussion

Recent studies have demonstrated that flavonoids have potent anti-tumor and anti-allergic activities as well as promising anti-HIV effects (Kitamura et al., 1998; Nair et al., 2002). Nobiletin has been reported to significantly lower blood pressure and reduce blood glucose levels in KKAY mice, as well as have inhibitory effects on production of oxygen radicals that are considered carcinogenic. Several studies have shown that nobiletin can reduce progression of colorectal cancer and cutaneous carcinoma in animal models (Kohno et al., 2001; Murakami et al., 2000a and b; Sato et al., 2002).
ANTI-HCV EFFECT OF CITRUS UNSHIU PEEL AND NOBILETIN

Figure 1. Inhibition of HCV infection in MOLT-4 cells by human serum treated with Citrus unshiu peel.

Figure 2. Inhibition of HCV infection in MOLT-4 cells by Citrus unshiu peel water extract.
Figure 3. Chemical structure of nobiletin.

Figure 4. (A) Inhibition of HCV infection in MOLT-4 cells by nobiletin. (B) Relative effects of nobiletin on eradication of HCV in peripheral blood of untreated patients. Data are shown as mean ± SD (n = 3). *P < 0.05, ***P < 0.001 versus controls.
As with gomisin A, nobiletin is chemically characterized by methylation of all hydroxyl groups. It has been demonstrated that 1′-acetoxychavicol acetate, auraptene and zerumbone, which have similar structures to nobiletin, also have anti-oxidant and anti-tumor effects. All these four substances may inhibit HCV infection. Further studies are needed to investigate whether nobiletin can be used as a new therapeutic agent in patients with chronic hepatitis C, and especially those who do not respond to IFN.

Acknowledgments
This work was supported in part by a Grant & Aid from Nihon Pharmaceutical University and Tsumura & Co. Tokyo, Japan.

References


Sato, T., L. Koike, Y. Miyata, M. Hirata, Y. Mimaki, Y. Sashida, M. Yano and A. Ito. Inhibition of activator protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a


Copyright of American Journal of Chinese Medicine is the property of World Scientific Publishing Company and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.