Antiviral Research 98 (2013) 44–53

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Oleanolic acid and ursolic acid: Novel hepatitis C virus antivirals that inhibit NS5B activity

Lingbao Kong [a,*,1], Shanshan Li [b,*,1], Qingjiao Liao [c], Yanni Zhang [a], Ruina Sun [a], Xiangdong Zhu [a], Qinghua Zhang [a], Jun Wang [a], Xiaoyu Wu [a], Xiaonan Fang [d], Ying Zhu [e]

[a] College of Bioscience and Engineering, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, China
[b] Department of Plant Pathology and Microbiology, Iowa State University, Ames, Iowa, IA 50011, USA
[c] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academic of Sciences, Wuhan, Hubei 430071, China
[d] Department of Immunology and Pathogen Biology, Tongji University School of Medicine, Shanghai 200092, China
[e] State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, China

ARTICLE INFO

Article history:
Received 29 October 2012
Revised 10 January 2013
Accepted 8 February 2013
Available online 16 February 2013

Keywords:
HCV
NS5B
Oleanolic acid
Ursolic acid
Antivirals

ABSTRACT

Hepatitis C virus (HCV) infects up to 170 million people worldwide and causes significant morbidity and mortality. Unfortunately, current therapy is only curative in approximately 50% of HCV patients and has adverse side effects, which warrants the need to develop novel and effective antivirals against HCV. We have previously reported that the Chinese herb *Fructus Ligustri Lucidi* (FLL) directly inhibited HCV NS5B RNA-dependent RNA polymerase (RdRp) activity (Kong et al., 2007). In this study, we found that the FLL aqueous extract strongly suppressed HCV replication. Further high-performance liquid chromatography (HPLC) analysis combined with inhibitory assays indicates that oleanolic acid and ursolic acid are two antiviral components within FLL aqueous extract that significantly suppressed the replication of HCV genotype 1b replicon and HCV genotype 2a JFH1 virus. Moreover, oleanolic acid and ursolic acid exhibited anti-HCV activity at least partly through suppressing HCV NS5B RdRp activity as noncompetitive inhibitors. Therefore, our results for the first time demonstrated that natural products oleanolic acid and ursolic acid could be used as potential HCV antivirals that can be applied to clinic trials either as monotherapy or in combination with other HCV antivirals.

1. Introduction

HCV infection is a serious worldwide problem that affects about 3% of the global population (Ip et al., 2012). Among those infected, approximately 20–30% develops liver disease, such as chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma. Current standard-of-care treatment consists of a combination of pegylated interferon-α (IFN-α) with ribavirin, which eradicates viruses in about 80% patients infected with HCV genotypes 2 or 3 (Ip et al., 2012; Liang et al., 2000; Zeuzem, 2008). However, only less than 50% of patients infected with HCV genotype 1 have been reported to successfully sustain an antiviral response, and most chronically infected patients remain untreated (Zeuzem, 2008). In 2011, two oral protease inhibitors, boceprevir and telaprevir, were approved by the US Food and Drug Administration (FDA) and applied to clinical treatment in combination with pegylated IFN and ribavirin. Although this combined therapy has significant improvements in viral eradication rates, these drugs induce adverse side effects and have reduced efficacy in patients who do not respond to standard-of-care treatment (Ip et al., 2012). Therefore, there is an urgent need for developing novel, more efficacious and safer anti-HCV drugs.

HCV is a positive-sense RNA virus of the family *Flaviviridae* with six major natural occurring genotypes (Lindenbach et al., 2005). Its genome consists of a 5′ untranslated region (UTR), a single open reading frame of ~9000 nucleotides in length and a short 3′UTR. The ORF encodes a single polyprotein that is cleaved by host and viral proteases into at least 10 mature viral proteins with the following order: NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Lindenbach and Rice, 2005). Among these viral proteins, NS5B is a RNA-dependent RNA polymerase (RdRp), a central enzyme in HCV RNA replication, and thus represents an attractive target for antiviral development (Behrens et al., 1996). Due to lack of robust infectious HCV cell culture system, for a long period only HCV replicon system was available for screening anti-HCV drugs until 2005 when three individual groups reported production of

* Corresponding authors. Tel.: +86 8791 83813459; fax: +86 8791 3828080 (L. Kong), tel.: +1515 203 9457; fax: +1515 203 9457 (S. Li).
E-mail addresses: lingbaok@hotmail.com (L. Kong), shanshanyxl@hotmail.com (S. Li), 6250831@qq.com (Q. Liao), zhang-yn99@hotmail.com (Y. Zhang), sunruina2009@163.com (R. Sun), Xdzhu815@yahoo.com.cn (X. Zhu), Zqh_net@163.com (Q. Zhang), 1807873529@qq.com (J. Wang), wuxiaoyu58@yahoo.com.cn (X. Wu), fxiaonan2004@yahoo.com.cn (X. Fang), yingzhu@whu.edu.cn (Y. Zhu).
[1] These authors contributed equally.

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.02.003

infectious HCV particles in cell culture from HCV genotype 2a (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). This HCV infection cell culture system makes it feasible to identify and develop novel antiviral drugs.

The traditional Chinese medicine, *Fructus Ligustri Lucidi* (FLL), is the dried ripen fruit of Ligustrum lucidum Ait and has been used as hepatoprotective agents for centuries (Committee of National Pharmacopoeia, 2000). We have previously reported that the aqueous FLL extract inhibits both the in vitro and intracellular HCV NS5B RdRp activity (Kong et al., 2007). However, little is known about whether the FLL aqueous extract has inhibitory effects on HCV replication as well as the active components that confer these inhibitory effects. In this study, we demonstrated that the FLL aqueous extract had strong anti-HCV activity and identified oleanolic acid and ursolic acid as the active components. Moreover, oleanolic acid and ursolic acid inhibited HCV replication at least partly through directly targeting HCV NS5B RdRp activity.

2. Materials and methods

2.1. Cells and plasmids

HepG2 cell line was obtained from China Centre for Type Culture Collection (Wuhan, China). Huh-7.5.1 cell line was kindly provided by Dr. Francis V. Chisari (The Scripps Research Institute, USA) (Zhong et al., 2005). 9–13 cell line harbouring a replicating HCV non-structural region with the use of the NS3-NS5B gene regions from the genotype 1b Con1 strain was kindly provided by Dr. Ralf Bartenschlager (University of Heidelberg, Germany) (Frese et al., 2002). Plasmid pJFH1 that contains the full-length HCV genotype 2a JFH1 strain cDNA was kindly provided by Dr. Takaji Wakita (National Institute of Infectious Diseases, Japan) (Wakita et al., 2005). The luciferase reporter plasmid pcDNA-(−)luc-(−)IRES for analyzing intracellular NS5B activity, and HepG2-5B cells that stably express wild-type HCV genotype 1a NS5B (derived from pBRTM/HCV1-3011 plasmid carrying full length ORF of HCV genotype 1a H strain) have been previously constructed (Kong et al., 2007). The JFH1 NS5B expression plasmids were constructed by inserting truncated NS5B sequence lacking the C-terminal 21 amino acids (NS5BΔ21, to ensure its solubility in *Escherichia coli* BL21 (DE3)) or full length NS5B sequence from plasmid pJFH1 into the pET–His and pcDNA3.1(−) vector, respectively. The primers used are listed in Supplementary Table 1. All hepatic cells used in this study were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) in a 5% CO_2/95% air humidified atmosphere. Cells that stably express NS5B were maintained in a similar medium with 200 μg/ml G418.

2.2. Preparation and isolation of FLL extract

Fructus Ligustri Lucidi (FLL) used in this study was purchased from Jiangxi Nanhua Medical and Pharmaceutical Co., Ltd (Nanchang, China). The aqueous FLL extract was prepared as described previously (Kong et al., 2007). The prepared aqueous FLL extract was then extracted with ethyl acetate for five times. Ethyl acetate-extracted fraction was then chromatographed on a silica gel TLC plate (Merck, Germany) and developed with a solvent mixture of chloroform: methanol in the ratio 18.5:1.5 (v/v). Five fractions named as fractions 1–5 were collected using ethyl acetate. These collected fractions were lyophilized using a Freeze Drier (Labconco, USA) and stored at −70 °C. Each lyophilized fraction was dissolved in DMEM with 2% dimethyl sulfoxide (DMSO, Sigma) immediately prior to the assays.

2.3. Other reagents

Oleanolic acid, ursolic acid and salidroside were purchased from National Institutes for Food and Drug Control (Beijing, China). Ligustroflavone and quercetin were purchased from Chengdu Mansite Bio-technology Co, Ltd (Chengdu, China). All these compounds were purchased with a purity ⩾98%. Oral oleanolic acid medication Qindunguosuanpian (China Drug Approval No. H20003499, the net content of oleanolic acid ≈26%) and oral ursolic acid medication Taizhian (China Drug Approval No. Z20000115, the net content of ursolic acid ≈11%) were purchased from Hunan Jiuzhitang Co, Ltd (Changsha, China) and China Resources Sanjiu Medical and Pharmaceutical Co., Ltd (Shenzhen, China), respectively. Ribavirin was purchased from Sigma–Aldrich (St. Louis, USA). Recombinant Human Interferon alfa-2b (IFNα-2b) was purchased from Anke-Bio (Hefei, China). Recombinant Human Interferon-gamma (IFN-γ) was purchased from ProSPec (East Brunswick, USA). The inhibitor of HCV NS5B RNA polymerase, 2′-O-Me-CTP, was purchased from Trilink (San Diego, USA). The inhibitors of HCV NS3/4A protease, ITMN-191 and MK-7009, were purchased from Axon Medchem BV (Groningen, Netherlands) and Merck (Whitehouse Station, USA), respectively. Each reagent was dissolved in DMEM with 2% DMSO during the assays.

2.4. Determination of antiviral activity in the JFH1 infection model

Plasmid pJFH1, containing the full-length cDNA of the HCV genotype 2a JFH1 isolate, was used to generate infectious HCV particles in Huh7.5.1 cell culture as described previously (Zhong et al., 2005). Cell culture media collected at day 10 posttransfection were centrifuged and passed through a 0.22 μm filter. The cell-free media were used as HCV JFH1 stocks, which were then aliquoted and stored at −80 °C.

Huh-7.5.1 cells were seeded in 24-well plates at a density of 3×10^4 cells/well one day before infection and then inoculated with 100 μl JFH1 virus. At 6 h postinfection, JFH1-infected cells were washed with fresh medium, treated with increasing concentrations of the tested reagents, and cultured for 2 days. The cells were harvested and divided into two equal groups. The first group was used to determine the intracellular viral RNA levels by real-time RT-PCR with HCV-specific RT primers (Supplementary Table 1) (Wu et al., 2011). The viral RNA levels were normalized to GAPDH RNA levels. The second group was used to determine HCV Core protein levels by Western blot using antibodies against HCV Core protein (C7-50, Abcam) and GAPDH (Thermo) as described previously (Wu et al., 2011).

2.5. Determination of antiviral activity in the HCV genotype 1b replicon model

9–13 cells were treated with increasing concentrations of the tested reagents, and cultured for 2 days. The cells were harvested and divided into two equal groups. The first group was used to determine the intracellular viral RNA levels by real-time RT-PCR with HCV genotype 1b NS5A and GAPDH specific primers as described previously (Hou et al., 2010) (Supplementary Table 1). The viral RNA levels were normalized to GAPDH RNA levels. The second group was used to determine HCV NS5A protein levels by Western blot using antibodies against HCV NS5A protein (Virogen) and GAPDH (Thermo) as described previously (Hou et al., 2010).

2.6. Cytotoxicity assays

The cytotoxic effects of tested reagents were measure by a MTT cell viability assay as described previously (Kong et al., 2007). The

reagent-untreated cells were assayed as controls (designated as 100% viability). All experiments were conducted in triplicate.

2.7. Luciferase reporter assay to determine intracellular NS5B activity

The inhibitory effects of tested reagents on intracellular NS5B activity was determined by a luciferase reporter assay as described previously (Kong et al., 2007). In brief, the reporter plasmid pcDNA-(−)luc-(−)IRES was digested with PstI. The digested large fragment (designated CLI), containing a CMV immediate-early promoter, a reverse luciferase gene followed by a cDNA copy of 3′ terminal region of the minus HCV RNA (complementary to the HCV 5′ UTR), was used for transfection. The HCV NS5B expressing cells, HepG2-5B cells stably expressing HCV genotype 1a NS5B or Huh7.5.1 cells transiently expressing HCV genotype 2a JFH1 NS5B, were co-transfected with CLI and pRL-CMV (used for normalization). Following incubation at 37 °C for 6 h, the medium was replaced by maintenance medium with different concentrations of the tested reagents. Cells were grown in the presence of the tested reagents for 2 days with daily medium exchanged, and were subsequently lysed with the Passive Lysis Buffer (Promega). Cell lysates were analyzed using the Dual-luciferase Reporter Assay system kit (Promega) according to the manufacturer's instructions. The firefly luciferase activity was normalized by referring to Renilla luciferase activity. All experiments were performed in triplicate.

2.8. Assessment of inhibitory effects of reagents on in vitro NS5B activity

The inhibitory effects of the tested reagents on in vitro NS5B activity was determined by NS5B-catalyzed RNA synthesis assay as described previously (Kong et al., 2007). Purified HCV genotype 1a NS5B protein and HCV (−) 3′ T RNA template were obtained as described previously (Ye et al., 2005). The hexahistidine-tagged HCV genotype 2a NS5B derived from JFH1 strain was expressed in *Escherichia coli* BL21 (DE3) and purified as described previously (Supplementary Fig. 1) (Ye et al., 2005). The tested reagents were added at a variety of concentrations in 50 μl reaction mixture consisting of 20 mM HEPES (pH 8.0), 1.5 mM $MnCl_2$, 100 mM ammonium acetate, 1 mM DTT, 500 μM GTP, 250 μM each of CTP, ATP and UTP, 40U of RNasin (Biostar, Canada), 2 μg/ml HCV (−) 3′ T RNA template and 300 ng purified NS5B protein. 2 μg 2′-O-Me-CTP was also tested as a positive control for inhibiting NS5B-catalyzed RNA synthesis. After 2 h at 30 °C, the reaction was stopped by 100 mM EDTA. The RNA products were extracted and subjected to Northern blot analysis as described previously (Kong et al., 2007).

2.9. Assessment of inhibitory mode of reagents on in vitro NS5B activity

To determine the mode of inhibition of NS5B polymerization activity by oleanolic acid and ursolic acid, the compounds were tested at a variety of concentrations (0–2.5 μg) in a final volume of 50 μl of reaction mixture consisting of 20 mM Tris–HCl (pH7.5), 5 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 300 ng of purified NS5B enzyme, 400 ng of poly(rA)/oligo$(dT)_{15}$ (Invitrogen), various concentrations (5–45 μM) of nonradioactive UTP, and 0.1–0.9 μCi of α-^{32}P-labeled UTP (3000 Ci/mmol, H-Y Biological, China). Reactions proceeded for 1 h at RT and were quenched by 100 mM EDTA. Radiolabelled RNA products were quantified using a liquid scintillation counter (Wallac 1450 MicroBeta TriLux; PerkinElmer Life Sciences) as previously described (Wang et al., 2003).

2.10. Analytical HPLC analysis

The five isolated FLL fractions, oleanolic acid, ursolic acid, salidroside, ligustroflavone and quercetin were analyzed by Waters Alliance 2690 HPLC system and C18 reversed-phase HPLC column. As these compounds cannot be separated in single chromatographic condition, we used different solvent systems, flow rates and test wavelengths for various reagents as described in Fig. 3 and Supplementary Fig. 3.

2.11. Assessment of inducing effects of interferon-β by oleanolic acid and ursolic acid

HepG2 cells were treated with increasing concentrations of oleanolic acid, ursolic acid or Poly (I:C) (Sigma). The cells were cultured for 2 days and harvested to determine the intracellular interferon-β mRNA levels by real-time RT-PCR with interferon-β and GAPDH specific primers (Supplementary Table 1). Interferon-β mRNA levels were normalized to GAPDH mRNA levels.

2.12. Statistical analyses

The method of relative quantification was used for analysis of the results of real-time RT-PCR, luciferase assay and cytotoxicity experiment. The effects of reagents were assessed by the percentage of values of reagent-treated groups relative to those of untreated control groups (% of control). Statistical analysis was performed using the Statistical Package Social Sciences (SPSS) program version 11.5 by one-way analysis of variance (ANOVA) and significant differences among groups were determined by Least Significant Difference (LSD). The accepted level of statistical significance was $p < 0.05$. 50% inhibitory concentration (IC50) and 50% cytotoxic concentration (CC50) were calculated using Statistical SPSS program version 11.5 by probit analysis of regression.

3. Results

3.1. Inhibition of HCV replication by isolated FLL fractions

The FLL aqueous extract was subjected to ethyl acetate extraction and TLC separation, yielding five fractions named as fractions 1–5 (Supplementary Fig. 2). To evaluate their antiviral activity against HCV replication, JFH1 virus-infected Huh-7.5.1 cells were treated with different concentrations of FLL fractions and JFH1 replication was monitored by measuring the viral RNA levels. As shown in Fig. 1, FLL fraction 1 and fraction 2 significantly reduced HCV RNA levels in a dose-dependent manner ($p < 0.05$); while the remaining three FLL fractions had no inhibitory activity even up to a concentration of 200 μg/ml. The IC50 values of fraction 1 and fraction 2 were 51.1 μg/ml and 11.9 μg/ml, respectively (Table 1). As a positive control, the IC50 value of 2′-O-Me-CTP was 24.1 μg/ml (Table 1). We also evaluated the cytotoxicity of five FLL fractions by MTT cell viability assays. Our data showed that the CC50 values of all fractions were more than 200 μg/ml and the CC50 of 2′-O-Me-CTP was above 100 μg/ml (Supplementary Table 2). These results indicated that isolated FLL fractions 1 and 2 inhibited JFH1 virus replication.

3.2. Inhibition of HCV NS5B activity by isolated FLL extracts

We have previously developed a luciferase reporter assay to measure the intracellular HCV NS5B activity by transfecting the reporter plasmid into the HCV NS5B-expressing cells and measuring the luciferase activity of tested cell lysates (Kong et al., 2007). Using this method, we hereby assessed the effects of the five iso-

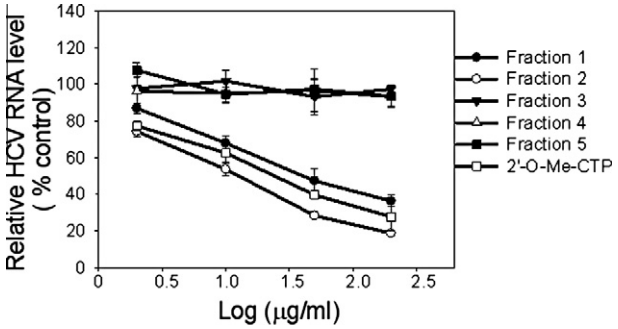

Fig. 1. Effects of isolated FLL fractions on JFH1 virus replication. Huh-7.5.1 cells were treated with different concentrations of FLL fractions 1–5, or 2′-O-Me-CTP for 2 days after being infected with JFH1 virus. The levels of intracellular HCV RNA were measured by real-time RT-PCR. The inhibitory effect was assessed by the percentage of values of reagent-treated groups relative to those of untreated control groups (% of control). Values represent means ± SD ($n = 3$).

Table 1
Concentrations of reagents that reduce intracellular HCV NS5B activity and replication by 50% (IC50).

Reagent	IC50 (μg/ml)			
	H strain NS5B	JFH1 NS5B	Genotype1b replicon	JFH1 virus
Fraction 1	33.8	NT	NT	51.1
Fraction 2	5.5	NT	NT	11.9
Fraction 3	>200	NT	NT	>200
Fraction 4	>200	NT	NT	>200
Fraction 5	>200	NT	NT	>200
2′-O-Me-CTP	8.4	7.2	NT	24.1
Oleanolic acid	0.8	1.0	3.5	2.9
Ursolic acid	3.1	6.4	19.2	10.6
Salidroside	>10	NT	NT	NT
Oral oleanolic acid	NT	NT	NT	21.1
Oral ursolic acid	NT	NT	NT	104.3
Ribavirin	NT	NT	NT	7.1
ITMN-191	NT	NT	NT	3.5×10^{-3}
MK-7009	NT	NT	NT	7.5×10^{-3}
IFNα-2b	NT	NT	NT	0.4 IU/ml
IFN-γ	NT	NT	0.64 IU/ml	NT

IC50, 50% inhibitory concentration; NT, not tested.

lated FLL fractions on intracellular NS5B activity using HepG2-5B cells that stably express wild-type HCV genotype 1a H strain NS5B. As shown in Fig. 2A, fraction 1 and fraction 2 inhibited the luciferase activity of tested cell lysates in a dose-dependent manner ($p < 0.001$), whereas fractions 3, 4 and 5 did not show any inhibitory activity within the tested doses. Moreover, the IC50 values of fraction 1 and fraction 2 were 33.8 μg/ml and 5.5 μg/ml, respectively (Table 1). As a positive control, the inhibitor of HCV NS5B, 2′-O-Me-CTP had an IC50 of 8.4 μg/ml (Table 1). The cytotoxicity assays showed that the CC50 values of all fractions were more than 200 μg/ml and the CC50 of 2′-O-Me-CTP was above 100 μg/ml (Supplementary Table 2). These results indicated that isolated FLL fractions 1 and 2 inhibited intracellular NS5B activity.

To directly test the inhibitory effects of the isolated FLL fractions on HCV NS5B RdRp activity, we employed in vitro NS5B-catalyzed RNA synthesis assay using purified HCV genotype 1a H strain NS5B. Both fraction 1 and fraction 2 inhibited the NS5B activity in a concentration-dependent manner (Fig. 2B). In contrast, fraction 3 (Fig. 2B), fractions 4 and 5 (data not shown) did not show dose-dependent inhibitory activity within the tested doses. Moreover, fraction 2 possessed higher inhibitory effect than fraction 1, consistent with their effects on intracellular NS5B activity

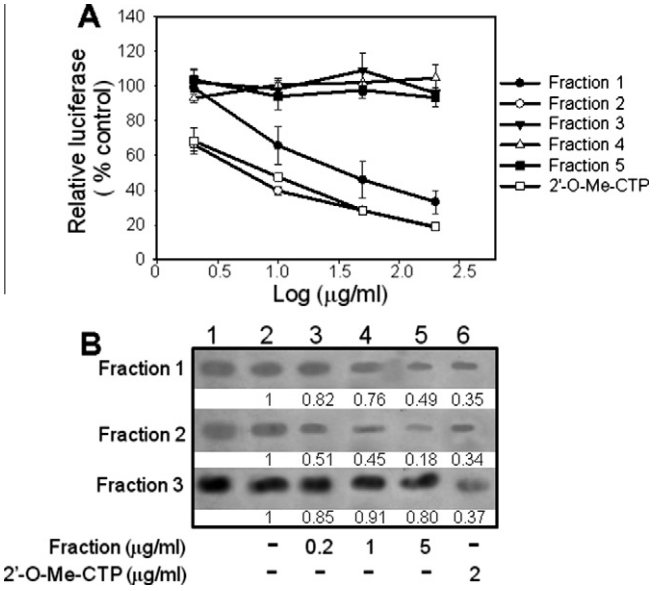

Fig. 2. Inhibition of HCV genotype 1a NS5B activity by isolated FLL fractions. (A) Effects of isolated FLL fractions on intracellular HCV genotype 1a NS5B activity. The HepG2-5B cells that stably express wild-type HCV genotype 1a H strain NS5B were transfected with CLI, treated with different concentrations of the isolated five FLL fractions and 2′-O-Me-CTP for 2 days, and then subjected to the luciferase assay. The inhibitory effect was assessed by the percentage of values of reagent-treated groups relative to those of untreated control groups (% of control). Values represent means ± SD ($n = 3$). (B) Effects of fractions 1 and 2 on in vitro HCV genotype 1a H strain NS5B activity. Lane 1, the RNAs transcribed from KpnI-linearized pGEM-(−)3′T with Sp6 RNA polymerase that were used as a RNA size marker; lanes 2–5, different concentrations of isolated FLL fraction 1 (upper panel) and fraction 2 (lower panel) treatments; lane 6, 2 μg/ml of 2′-O-Me-CTP treatment. The relative intensity of each blot was indicated by numbers below.

(Fig. 2). As a positive control, 2 μg/ml of 2′-O-Me-CTP significantly reduced NS5B RdRp activity (Fig. 2B). These results suggest that the isolated FLL fraction 1 and fraction 2 directly inhibited NS5B RdRp activity with fraction 2 more potent than fraction 1 and this inhibitory effect might explain their antiviral activity.

3.3. HPLC analysis of the isolated FLL extracts

To identify the active components that inhibit HCV replication, we used analytical HPLC to analyze the FLL fractions. Five reported main constituents in FLL extract, oleanolic acid, ursolic acid, salidroside, ligustroflavone and quercetin were also used for HPLC analysis as standard controls (Fig. 3 and Supplementary Fig. 3). Compared to other FLL fractions, fraction 1 has one distinct main peak (44.8% of total peak area), which is consistent with ursolic acid under the same HPLC condition (Fig. 3B). Fraction 2 has one distinct peak (18.3% of total peak area), which is consistent with oleanolic acid under the same HPLC condition (Fig. 3A). However, fractions 1 and 2 do not have a great amount of other compounds including salidroside, ligustroflavone and quercetin (Supplementary Fig. 3). These data implied that oleanolic acid and ursolic acid might be the active antiviral components.

3.4. Inhibition of HCV replication by oleanolic acid, ursolic acid and related oral medications

To evaluate the effects of oleanolic acid and ursolic acid on HCV replication, we first treated JFH1-infected cells with different concentrations of oleanolic acid, ursolic acid, oral oleanolic acid medication, oral ursolic acid medication. Meanwhile, the inhibitors of HCV NS3/4A protease (ITMN-191 and MK-7009) and the known

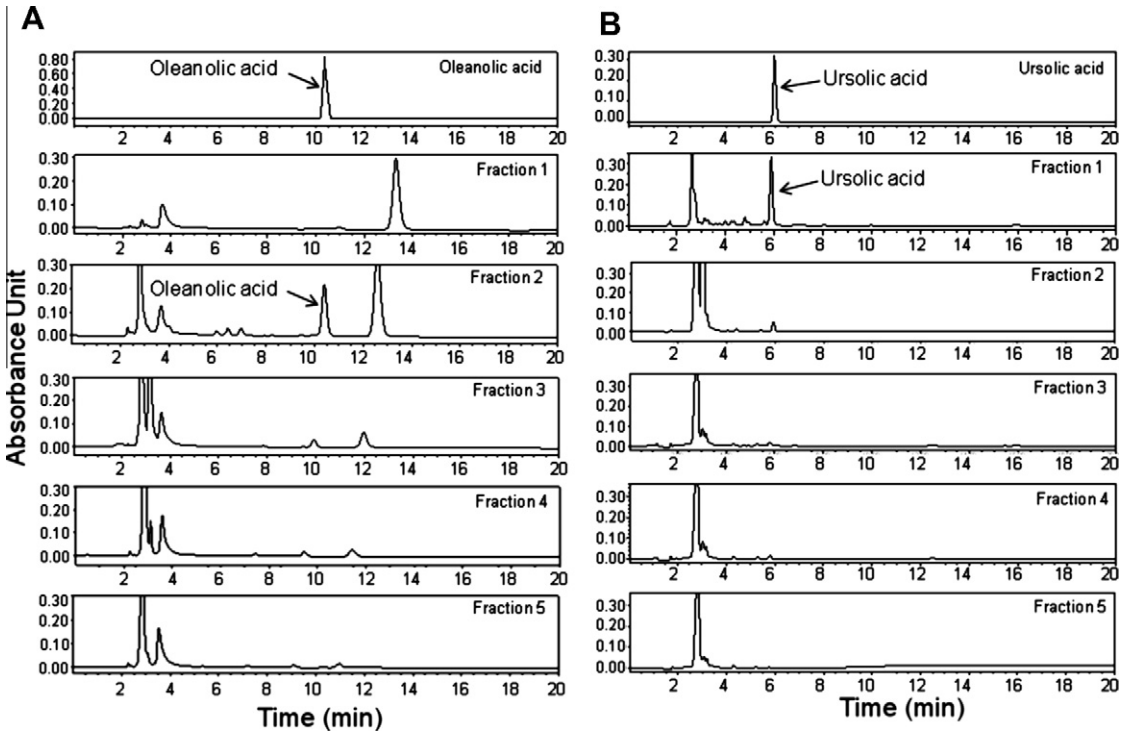

Fig. 3. HPLC analysis of isolated FLL fractions. (A) The isolated FLL fractions 1–5 as well as oleanolic acid were analyzed by Waters Alliance 2690 HPLC system and C18 reversed-phase HPLC column. Mobile phase consisted of a solvent mixture of methanol:water:ethylic acid:triethylamine in the ratio 87:13:0.04:0.02(v/v). The flow rate and test wavelength were 1 ml/min and 210 nm, respectively. (B) The isolated FLL fractions 1–5 as well as ursolic acid were analyzed by Waters Alliance 2690 HPLC system and C18 reversed-phase HPLC column. Mobile phase consisted of a solvent mixture of methyl cyanide: 0.5% ethylic acid in the ratio 90:10 (v/v). The flow rate and test wavelength were 0.8 ml/min and 210 nm, respectively. The elute peaks corresponding to oleanolic acid and ursolic acid were indicated by arrows.

HCV antivirals (ribavirin and IFNα-2b) were used as positive controls. The HCV replication was monitored by measuring the expression levels of intracellular HCV RNA and Core protein. As shown in Fig. 4A, oleanolic acid, ursolic acid, oral oleanolic acid medicine, oral ursolic acid medicine significantly reduced HCV RNA levels in a dose-dependent manner ($p < 0.05$); while salidroside did not show inhibitory activity even up to a concentration of 50 μg/ml. Moreover, oleanolic acid possessed higher inhibitory activity than ursolic acid in the same test doses (Fig. 4A). The IC50 values of oleanolic acid, ursolic acid, oral oleanolic acid medicine, oral ursolic acid medicine, ITMN-191, MK-7009, ribavirin and IFNα-2b were 2.9 μg/ml, 10.6 μg/ml, 21.1 μg/ml, 104.3 μg/ml, 3.5×10^{-3} μg/ml, 7.5×10^{-3} μg/ml, 7.1 μg/ml and 0.4 IU/ml, respectively (Table 1). Western blot analysis showed that oleanolic acid and ursolic acid strongly reduced the expression of HCV Core protein in a dose-dependent manner and salidroside did not show inhibitory activity even up to a concentration of 25 μg/ml (Fig. 4B). The CC50 values of oleanolic acid, ursolic acid, oral oleanolic acid medicine, oral ursolic acid medicine, ITMN-191, MK-7009, ribavirin and IFNα-2b against Huh7.5.1 cells were 89.2 μg/ml, 71.1 μg/ml, >200 μg/ml, >200 μg/ml, 47.4 μg/ml, 133.5 μg/ml, 81.4 μg/ml, above 10^4 IU/ml, respectively (Supplementary Table 2), indicating that these compounds had no or low cytotoxicity at tested inhibitory concentrations.

We also evaluated the effects of oleanolic acid and ursolic acid on HCV genotype 1b replication. 9–13 cells harbouring HCV genotype 1b replicon were treated with different concentrations of oleanolic acid and ursolic acid with IFN-γ as a positive control (Frese et al., 2002). As shown in Fig. 5A, oleanolic acid and ursolic acid significantly reduced HCV RNA levels in a dose-dependent manner ($p < 0.05$). The IC50 values of oleanolic acid, ursolic acid and IFN-γ were 3.5 μg/ml, 19.2 μg/ml, and 0.64 IU/ml, respectively (Table 1). Western blot analysis showed that oleanolic acid, ursolic acid and IFN-γ strongly reduced the expression of HCV NS5A protein (Fig. 5B). The CC50 values of oleanolic acid, ursolic acid and IFN-γ were 78.6 μg/ml, 58.4 μg/ml, and above 10^4 IU, respectively (Supplementary Table 2), indicating that these compounds had no or low cytotoxicity at tested inhibitory concentrations.

3.5. Inhibition of HCV NS5B activity by oleanolic acid and ursolic acid

To explore the antiviral mechanism of oleanolic acid and ursolic acid, we evaluated their effects on HCV NS5B activity. For HCV genotype 1a NS5B, the luciferase reporter assays showed that oleanolic acid, ursolic acid and 2′-O-Me-CTP inhibited the luciferase activity of tested HepG2-5B cells in a dose-dependent manner ($p < 0.001$) (Fig. 6A). The IC50 values of oleanolic acid, ursolic acid and 2′-O-Me-CTP were 0.8 μg/ml, 3.1 μg/ml and 8.4 μg/ml, respectively, confirming that oleanolic acid has more potent inhibitory activity than ursolic acid (Table 1). In contrast, salidroside showed no inhibitory activity up to a concentration of 10 μg/ml (Fig. 6A). The cytotoxicity assays indicated that the CC50 values of oleanolic acid and ursolic acid against HepG2-5B cells were more than 100 μg/ml (Supplementary Table 2).

Oleanolic acid and ursolic acid were then tested for their effects on in vitro HCV genotype 1a NS5B activity by in vitro NS5B-catalyzed RNA synthesis assay. As shown in Fig. 6B, oleanolic acid, ursolic acid and inhibited HCV genotype 1a NS5B activity in a dose-dependent manner. In contrast, salidroside did not show inhibitory activity up to a concentration of 2.5 μg/ml (data not shown). As a positive control, 2.0 μg/ml of 2′-O-Me-CTP reduced in vitro NS5B activity. These results suggest that oleanolic acid and ursolic acid inhibited both the intracellular and in vitro activity of HCV genotype 1a NS5B.

We also examined the effects of these two compounds on the HCV genotype 2a JFH1 NS5B activity. As shown in Fig. 6C, oleanolic

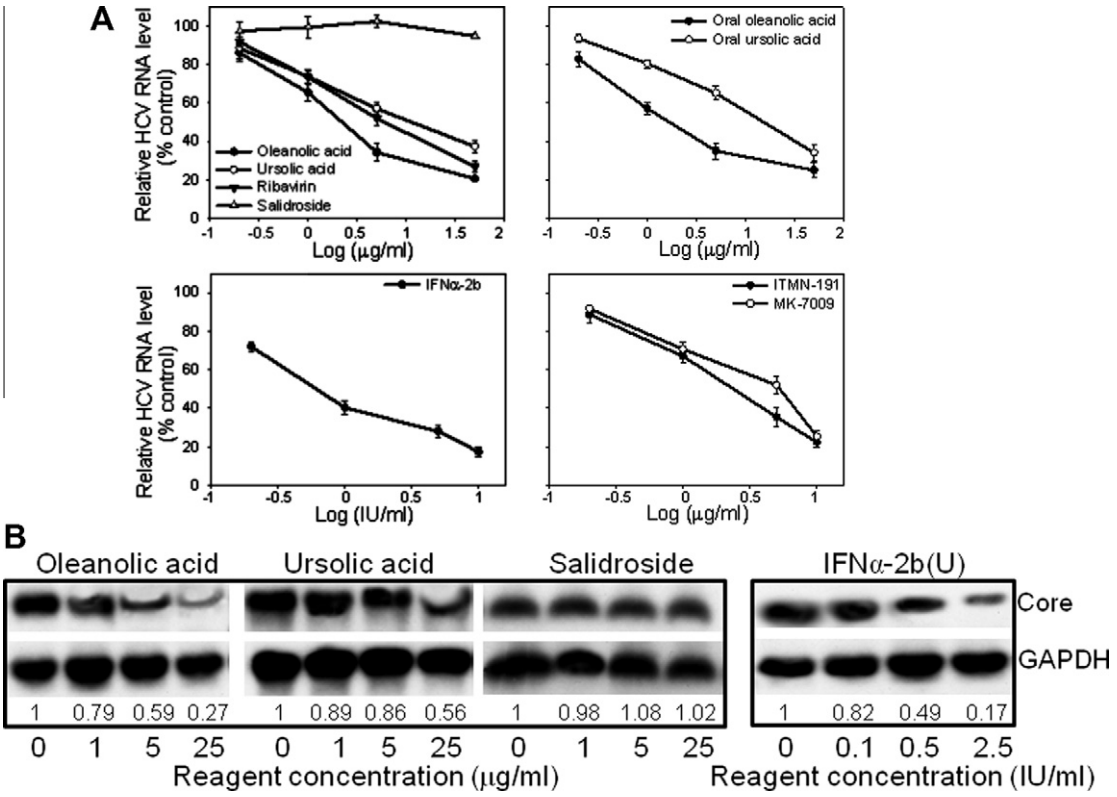

Fig. 4. Inhibition of JFH1 virus replication by oleanolic acid, ursolic acid and related oral medications. (A) Huh-7.5.1 cells were treated with different concentrations of oleanolic acid, ursolic acid, salidroside, oral oleanolic acid medication, oral ursolic acid medication, ITMN-191, MK-7009, ribavirin or IFNα-2b for 2 days after being infected with JFH1 virus. The levels of intracellular HCV RNA were measured by real-time RT-PCR. (B) JFH1-infected Huh-7.5.1 cells were treated with different concentrations of oleanolic acid, ursolic acid, salidroside, or IFNα-2b for 2 days. Cell lysates were separated by SDS–PAGE, followed by Western blot analysis with antibodies against HCV Core protein and GAPDH. The relative intensity of each blot after normalization to that of GAPDH was indicated by numbers below.

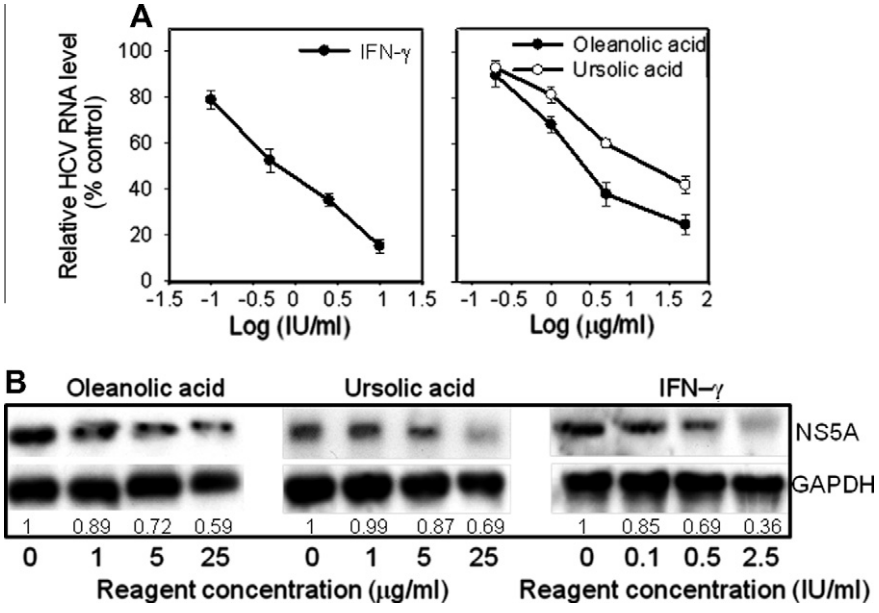

Fig. 5. Inhibition of HCV genotype 1b replicon by oleanolic acid, ursolic acid and IFN-γ. 9–13 cells were treated with increasing concentrations of oleanolic acid, ursolic acid, or IFN-γ for 2 days. (A) Analysis of intracellular HCV RNA levels by real-time RT-PCR. (B) Western blot analysis of cell lysate with antibodies against HCV NS5A protein and GAPDH. The relative intensity of each blot after normalization to that of GAPDH was indicated by numbers below.

acid and ursolic acid inhibited the intracellular JFH1 NS5B activity in a dose-dependent manner ($p < 0.001$). The IC50 values of oleanolic acid and ursolic acid were 1.0 μg/ml and 6.4 μg/ml, respectively. As a positive control, the IC50 of 2′-O-Me-CTP was 7.2 μg/ml (Table 1). As described above, the CC50 values of oleanolic acid, ursolic acid and 2′-O-Me-CTP against Huh7.5.1 cells were 89.2 μg/ml, 71.1 μg/ml and above 100 μg/ml, respectively (Supplementary Table 2). Oleanolic acid and ursolic acid were then tested for their effect on in vitro JFH1 NS5B activity by in vitro NS5B-catalyzed RNA synthesis assay. As shown in Fig. 6D, oleanolic acid and ursolic

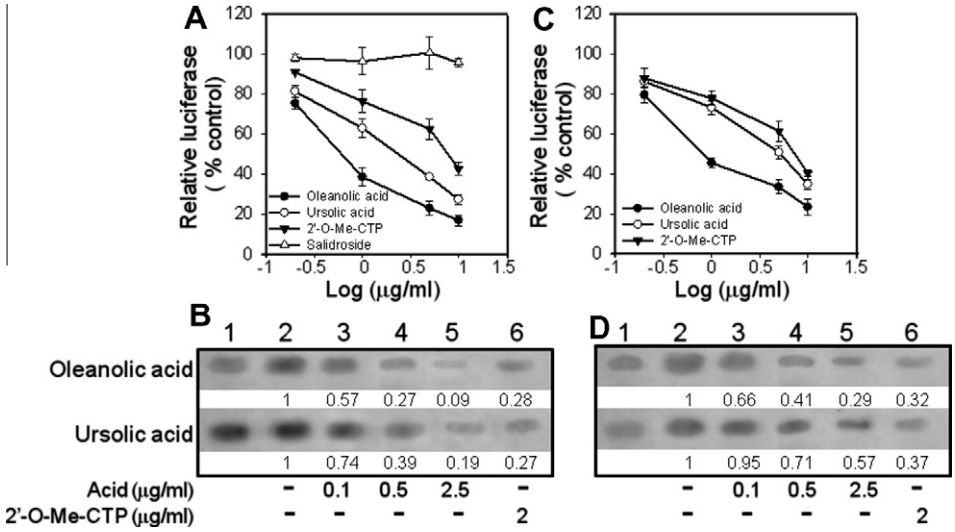

Fig. 6. Inhibition of HCV NS5B activity by oleanolic acid and ursolic acid. (A and C) Effects of oleanolic acid and ursolic acid on intracellular NS5B activity. HepG2-5B cells stably expressing wild-type HCV genotype 1a H strain NS5B (A) or Huh7.5.1 cells that transiently expressing HCV genotype 2a JFH1 NS5B (C) were transfected with CLI, treated with different concentrations of oleanolic acid, ursolic acid, 2′-O-Me-CTP and salidroside for 2 days, and then subjected to the luciferase assay. (B and D) Effects of oleanolic acid and ursolic acid on in vitro activity of NS5B from HCV genotype 1a (B) or HCV genotype 2a JFH1 (D). Lane 1, the RNA size marker; lanes 2–5, different concentrations of oleanolic acid (upper panel) or ursolic acid (lower panel) treatments; lane 6, 2 μg/ml of 2′-O-Me-CTP treatment. The relative intensity of each blot was indicated by numbers below.

acid inhibited JFH1 NS5B activity in a dose-dependent manner. As a positive control, 2 μg of 2′-O-Me-CTP significantly reduced NS5B activity.

To gain further insight into the mechanism for how oleanolic acid and ursolic acid inhibit HCV NS5B, we examined their inhibitory effects on NS5B polymerization activity by measuring the incorporation of ^{32}P-labelled UTP into a newly synthesized RNA using an poly(rA)/oligo(dT)$_{15}$ as a homopolymeric template/primer in the presence of different concentrations of oleanolic acid or ursolic acid. As shown in Fig. 7, oleanolic acid and ursolic acid were found to act as noncompetitive inhibitors with respect to the UTP substrate with Ki values of ~2.5 μg/ml and ~4.7 μg/ml for HCV genotype 1a H strain NS5B, ~3.7 μg /ml and ~5.8 μg /ml for HCV genotype 2a JFH1 NS5B, respectively.

3.6. Oleanolic acid and ursolic acid have no inducing effect of interferon-β in HepG2 cells

Pentacyclic triterpenoid, ME3738, has been reported to inhibit HCV replication through activating interferon-β pathway in HepG2 cells (Hiasa et al., 2008). As oleanolic acid and ursolic acid belong to pentacyclic triterpenoid compounds (Fig. 8A), we tested whether these two compounds could induce interferon-β pathway by measuring the levels of interferon-β mRNA via real-time RT-PCR. As shown Fig. 8B, both oleanolic acid and ursolic acid showed no inducing effect on interferon-β transcription in HepG2 cells. In contrast, poly(I:C), as a positive control (Jefferies and Fitzgerald, 2005), significantly induced interferon-β mRNA in tested doses.

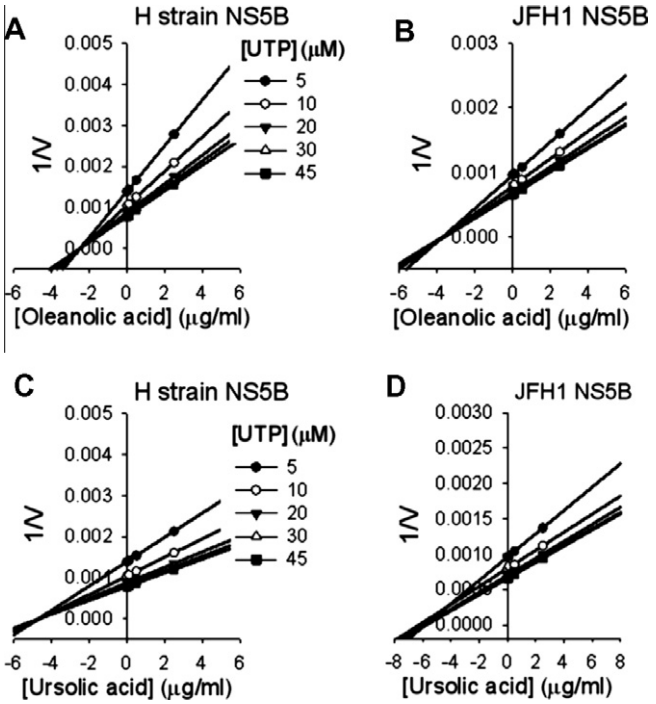

Fig. 7. Dixon plot of the inhibition of NS5B polymerase in the presence of oleanolic acid (A and B) or ursolic acid (C and D). The activity of HCV genotype 1a H strain NS5B (A and C) and JFH1 NS5B (B and D) was measured using poly(rA)/oligo(dT)$_{15}$ as template/primer. The reactions were performed in the presence of increasing concentrations of UTP substrate (5–45 μM) and increasing concentrations (0–2.5 μg) of inhibitors. Values represent means ± SD (n = 3).

4. Discussion

Chronic HCV infection remains a serious burden on public health worldwide; however, there are no protective HCV vaccines and effective broad-spectrum antiviral therapies available for all genotypes of HCV, which prompted medical researchers around the world to identify novel and effective antiviral agents (Assis and Lim, 2012). With people's efforts to elucidate the mechanisms of HCV replication and pathogenesis, it has been proposed that HCV proteins essential for viral replication and infection are potential antiviral targets (Rice, 2011). To develop HCV specific therapy, extensive attempts have been focused on HCV viral proteins including NS3-4A serine protease, NS3 RNA helicase, NS5A, NS5B RNA-dependent RNA polymerase (Assis and Lim, 2012; Rehman et al., 2011). Recent advances indicate that NS4B is also a promising target for antiviral treatments (Li et al., 2012; Rai and Deval, 2011). In the meantime, several HCV inhibitors targeting these vir-

al proteins have been synthesized or identified and some of them have been applied into different phases of clinical trials (Assis and Lim, 2012). Two oral protease inhibitors, boceprevir and telaprevir, have been approved by the US FDA (Ip et al., 2012). Unfortunately, HCV undergoes rapid mutations to generate variants that are resistant to these direct-acting antiviral (DAA) molecules (Pawlotsky, 2011). Thus, there is a continuous need to develop new potential antiviral drugs from other sources such as natural medicinal herbs, which have been used as antiviral drugs for many years in many regions (Ahmed-Belkacem et al., 2010; Hudson, 1989; Morishima et al., 2010). As a RNA-dependent RNA polymerase (RdRp), NS5B has no counterpart in mammalian cells and thus it is conceivable that inhibition of this enzyme could not cause target-related side effects (De Re, 2010). We have previously shown that aqueous FLL extract inhibits both intracellular and in vitro NS5B RdRp activity (Kong et al., 2007). In the present study, we further examined the antiviral activity of aqueous FLL extract against HCV and analyzed its active antiviral components.

The ethyl acetate fraction of the aqueous FLL extract was separated to five fractions on a silica gel TLC plate (Supplementary Fig. 2). Among these five fractions, only fractions 1 and 2 reduced intracellular JFH1 RNA levels in a dose-dependent manner (Fig. 1) and the other three FLL fractions had no inhibitory activities, indicating that the active component(s) may be abundant in fractions 1 and 2. In addition, fraction 2 had a significant lower IC50 (11.9 μg/ml) than that (51.1 μg/ml) of fraction 1 (Table 1), indicating that fraction 2 possessed higher antiviral activity than fraction 1. Furthermore, FLL fraction 1 and fraction 2 dose-dependently inhibited both in vitro and intracellular HCV genotype 1a NS5B activity and fraction 2 had higher anti-NS5B activity (Fig. 2), implying that the antiviral activity of FLL fraction 1 and 2 might be due to their inhibitory effects on HCV NS5B activity.

To identify the active antiviral component(s), we employed HPLC to analyze the isolated FLL fractions with five known FLL components (oleanolic acid, ursolic acid, salidroside, ligustroflavone and quercetin) as standard controls. Compared to other FLL fractions, both fractions 1 and 2 have one distinct main peak, which corresponds to that of ursolic acid and oleanolic acid, respectively, implying that these two compounds may be the antiviral components (Fig. 3). Our inhibition assays demonstrated that oleanolic acid and ursolic acid significantly repressed the replication of HCV genotype 2a JFH1 and HCV genotype 1b replicon (Figs. 4 and 5), implying their broad-spectrum antiviral effects. It is noticeable that there is one additional unknown peak in fraction 2 (Fig. 3A) and this peak might contain antiviral compound(s). Future efforts are required to address this question. To explore the possible antiviral mechanism of oleanolic acid and ursolic acid, we examined their effects on HCV NS5B activity. As expected, oleanolic acid and ursolic acid inhibited both the intracellular and in vitro HCV genotype 1a NS5B activity and oleanolic acid possesses higher inhibitory activity than ursolic acid in the same test doses (Fig. 6A and B). In addition to inhibiting HCV genotype 1a NS5B activity, oleanolic acid and ursolic acid have been shown to inhibit the activity of HCV genotype 2a JFH1 NS5B (Fig. 6C and D), despite the fact that the NS5B sequence identity of these two strains is only 73% by BLAST. It has been reported that amino acid substitutions in NS5B can easily confer resistance to NS5B-specific inhibitors (Pawlotsky, 2011). Our data suggest that these two compounds may have several target sites in NS5B and thus may have broad antiviral activity. Like some NS5B non-nucleoside analogue inhibitors (Wang et al., 2003), oleanolic acid and ursolic acid function as noncompetitive inhibitors of NS5B RdRp activity (Fig. 7), indicating that these two compounds could bind to NS5B independent of its substrate. Collectively, these results suggest that the

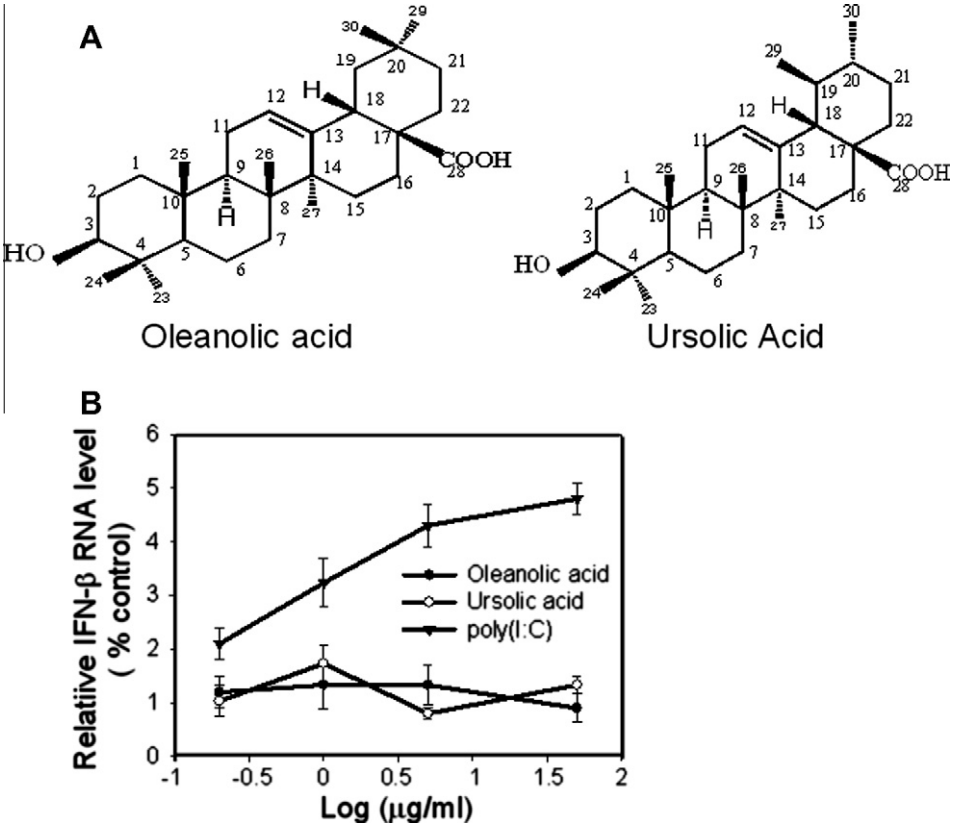

Fig. 8. Oleanolic acid and ursolic acid have no effect on interferon-β expression in HepG2 cells. (A) Chemical formulae of oleanolic acid and ursolic acid. (B) Real-time RT-PCR analysis of interferon- β mRNA in HepG2 cells in the presence of different concentrations of oleanolic acid, ursolic acid or poly(I:C). Values represent means ± SD (n = 3).

broad-spectrum antiviral activity of oleanolic acid and ursolic acid was mediated at least partly by directly targeting HCV NS5B RdRp activity.

Intriguingly, we observed that the inhibitory effects of oleanolic acid and ursolic acid for in vitro NS5B activity, intracellular NS5B activity and HCV virus replication gradually decrease in the same tested doses (Figs. 4–6 and Table 1). For instance, the IC50 values of oleanolic acid against in vitro and intracellular activity of HCV genotype 2a JFH1 NS5B were ~0.5 μg/ml and 1.0 μg/ml, respectively, whereas its IC50 values against the replication of JFH1 and HCV genotype 1b replicon were 2.9 μg/ml and 3.5 μg/ml. This phenomenon may be due to the intrinsic technique differences or the interference from certain components in the last two experimental systems such as host and viral proteins. HCV NS5B has been reported to interact with several cellular proteins such as p68, eIF4AII, protein kinase C-related kinase 2, a SNARE-like protein, nucleolin, and cyclin A2 (Kong et al., 2007; Pham et al., 2012). In addition, NS5B interacts with other HCV non-structural proteins to comprise the high-order replication complex during HCV replication (Waris et al., 2004). It is highly likely that these interactions might help stabilize the NS5B activity or interfere with the interaction between NS5B and its inhibitors (oleanolic acid and ursolic acid).

For centuries, the Chinese herb, *Fructus Ligustri Lucidi* (FLL) has been used for medicine purposes due to its hepatoprotective, immunomodulatory, anti-inflammatory, anti-tumor and anti-aging activities (Committee of Chinese Materia Medica, 1999; Committee of National Pharmacopoeia, 2000). A variety of physiologically active compounds have been identified in FLL including oleanolic acid, ursolic acid, ligustroside, quercetin, salidroside, etc. (Lin et al., 2007; Committee of Chinese Materia Medica, 1999; Committee of National Pharmacopoeia, 2000). Both oleanolic acid and ursolic acid are pentacyclic triterpenoid compounds (Fig. 8A) and have been approved as hepatoprotective agents by China Food and Drug Administration for many years. Accumulating evidence indicates that oleanolic acid and ursolic acid also exhibit anti-inflammatory, anti-tumor, anti-HIV or hypolipidemic activities (Bachhav et al., 2011; Chen et al., 2007; Fujiwara et al., 2011; Martin-Aragon et al., 2001; Mengoni et al., 2002). In this study, oleanolic acid and ursolic acid were found to possess anti-HCV activity. Unlike pentacyclic triterpenoid ME3738, which inhibits HCV replication through enhancing interferon-β in HepG2 cells (Hiasa et al., 2008), oleanolic acid and ursolic acid have no effect on interferon-β pathway (Fig. 8B), which reflected their structural differences and implied that these two compounds exert anti-HCV activity probably not through enhancing host antiviral activity but through directly interfering with HCV replication. Based on their isomeric structures (Fig. 8A), the last ring in pentacyclic triterpenoid compounds might be important for their inhibitory activity as the less potent ursolic acid has one CH_3 branched to last ring at C-19 position instead of C-20 position compared with oleanolic acid. This information may be valuable for us to improve their antiviral activity by modifying these compounds.

Table 2
Selectivity index of reagents against HCV JFH1 virus replication.

Reagent	IC50 (μg/ml)	CC50 (μg/ml)	SI (CC50/IC50)
Oleanolic acid	2.9	89.2	30.8
Ursolic acid	10.6	71.1	6.7
Oral oleanolic acid	21.1	>200	>9.5
Oral ursolic acid	104.3	>200	>1.9
2′-O-Me-CTP	24.1	>100	>4
Ribavirin	7.1	47.4	6.7
ITMN-191	3.5×10^{-3}	133.5	3.8×10^4
MK-7009	7.5×10^{-3}	81.4	1.1×10^4
IFNα-2b	0.4 U	$>10^4$ U	$>2.5 \times 10^4$

IC50, 50% inhibitory concentration; CC50, 50% cytotoxic concentration; SI, Selectivity index.

The Selectivity index (SI) values of oleanolic acid and ursolic acid against JFH1 virus replication are 30.8 and 6.7, which are significantly lower than that of HCV NS3/4A protease inhibitors, ITMN-191 (SI = 3.8×10^4) and MK-7009 (SI = 1.1×10^4) (Table 2). The SI values of these two compounds are also far smaller than that of current anti-HCV drug IFNα-2b (above 2.5×10^4) and have the same order of magnitude with that of anti-HCV drug ribavirin (SI = 6.7) (Table 2), implying their potential usage as anti-HCV drugs. We are pleased to see that oral oleanolic acid and ursolic acid medication significantly inhibit HCV replication (Fig. 4A). Therefore, our data suggest that both oleanolic acid and ursolic acid could be used as adjuvant or alternative drugs against HCV.

HCV is a highly variable virus and has six major natural occurring genotypes, which are further subdivided into a variety of subtypes (Lindenbach et al., 2005). This enormous variability renders the fact that many HCV-targeted drugs exhibit the viral genotype-specific differences in efficacy and the rapid evolution of drug-resistant mutants. For example, the current pegylated IFN-α and ribavirin combined treatment regimen cured about 80% of patients infected with HCV genotypes 2 or 3 but only 40%-50% of individuals infected with HCV genotypes 1 or 4 (Ahmed-Belkacem et al., 2010). Therefore, it is important to optimize anti-HCV drug candidates through studying their spectrum of action on the different genotypes as well as their resistance profile. The HCV genotype 1 strains are the most prevalent genotypes associated with liver disease and it also belongs to one of difficult-to-treat HCV strains (Rai and Deval, 2011). In this study, we showed that oleanolic acid and ursolic acid not only suppressed the replication of HCV genotype 2a JFH1 virus, but also inhibited the replication of HCV genotype 1b replicon, suggesting the ability of these two compounds to inhibit the replication of different HCV genotypes.

In summary, we found that FLL aqueous extract strongly suppressed HCV replication in part by inhibiting HCV NS5B RdRp activity. Moreover, we have identified two pentacyclic triterpenoid compounds, oleanolic acid and ursolic acid as the active antiviral compounds in FLL extract that inhibited both HCV replication and HCV NS5B RdRp activity. Our results provide a basis for optimization and subsequent development of pentacyclic triterpenoid derivatives as specific HCV antivirals. The combination of oleanolic acid and ursolic acid with pegylated IFNα and ribavirin could be useful in treatment of difficult-to-treat populations such as patients of HCV genotype 1b. Further efforts are required to characterize the underlie mechanisms and identify the target sites of oleanolic acid and ursolic acid on HCV NS5B.

Acknowledgements

This work was funded by research grants from National Nature Science Foundation of China (No. 31160034) to L. Kong, and Jiangxi Province (Nos. 2009BNA09400, 2010GZY0059 and GJJ12219) to L. Kong.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.02.003.

References

Ahmed-Belkacem, A., Ahnou, N., Barbotte, L., Wychowski, C., Pallier, C., Brillet, R., Pohl, R.T., Pawlotsky, J.M., 2010. Silibinin and related compounds are direct inhibitors of hepatitis C virus RNA-dependent RNA polymerase. Gastroenterology 138, 1112–1122.
Assis, D.N., Lim, J.K., 2012. New pharmacotherapy for hepatitis C. Clin. Pharmacol. Ther. 92, 294–305.

Bachhav, S.S., Patil, S.D., Bhutada, M.S., Surana, S.J., 2011. Oleanolic acid prevents glucocorticoid-induced hypertension in rats. Phytother. Res. 25, 1435–1439.
Behrens, S.E., Tomei, L., De Francesco, R., 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. 15, 12–22.
Chen, L., Zhang, Y., Kong, X., Peng, S., Tian, J., 2007. Synthesis and biological evaluation of nitric oxide-releasing derivatives of oleanolic acid as inhibitors of HepG2 cell apoptosis. Bioorg. Med. Chem. Lett. 17, 2979–2982.
Committee of Chinese Materia Medica, 1999. Chinese Materia Medica, vol. 6. Shanghai Science and Technology Press, pp. 183–189.
Committee of National Pharmacopoeia, 2000. Pharmacopoeia of PR China. Press of Chemical Industry, Beijing, p. 34.
De Re, V., 2010. Interferon-based therapy for chronic hepatitis C: current and future perspectives. Hepat. Mon. 10, 231–232.
Frese, M., Schwarzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O., Bartenschlager, R., 2002. Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology 35, 694–697.
Fujiwara, Y., Komohara, Y., Kudo, R., Tsurushima, K., Ohnishi, K., Ikeda, T., Takeya, M., 2011. Oleanolic acid inhibits macrophage differentiation into the M2 phenotype and glioblastoma cell proliferation by suppressing the activation of STAT3. Oncol. Rep. 26, 1533–1537.
Hiasa, Y., Kuzuhara, H., Tokumoto, Y., Konishi, I., Yamashita, N., Matsuura, B., Michitaka, K., Chung, R.T., Onji, M., 2008. Hepatitis C virus replication is inhibited by 22β-methoxyolean-12-ene-3β, 24(4β)-diol (ME3738) through enhancing interferon-β. Hepatology 48, 59–69.
Hou, W., Tian, Q., Zheng, J., Bonkovsky, H.L., 2010. MicroRNA-196 represses Bach1 protein and hepatitis C virus gene expression in human hepatoma cells expressing hepatitis C viral proteins. Hepatology 51, 1494–1504.
Hudson, J.B., 1989. Plant photosensitizers with antiviral properties. Antiviral Res. 12, 55–74.
Ip, P.P., Nijman, H.W., Wilschut, J., Daemen, T., 2012. Therapeutic vaccination against chronic hepatitis C virus infection. Antiviral Res. 96, 36–50.
Jefferies, C.A., Fitzgerald, K.A., 2005. Interferon gene regulation: not all roads lead to Tolls. Trends Mol. Med. 11, 403–411.
Kong, L., Li, S., Han, X., Xiang, Z., Fang, X., Li, B., Wang, W., Zhong, H., Gao, J., Ye, L., 2007. Inhibition of HCV RNA-dependent RNA polymerase activity by aqueous extract from *Fructus Ligustri Lucidi*. Virus Res. 128, 9–17.
Li, S., Yu, X., Guo, Y., Kong, L., 2012. Interaction networks of hepatitis C virus NS4B: implications for antiviral therapy. Cell. Microbiol. 14, 994–1002.
Liang, T.J., Rehermann, B., Seeff, L.B., Hoofnagle, J.H., 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann. Intern. Med. 132, 296–305.
Lin, H.M., Yen, F.L., Ng, L.T., Lin, C.C., 2007. Protective effects of Ligustrum lucidum fruit extract on acute butylated hydroxytoluene-induced oxidative stress in rats. J. Ethnopharmacol. 111, 129–136.
Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309, 623–626.
Lindenbach, B.D., Rice, C.M., 2005. Unravelling hepatitis C virus replication from genome to function. Nature 436, 933–938.
Martin-Aragon, S., de las Heras, B., Sanchez-Reus, M.I., Benedi, J., 2001. Pharmacological modification of endogenous antioxidant enzymes by ursolic acid on tetrachloride-induced liver damage in rats and primary cultures of rat hepatocytes. Exp. Toxicol. Pathol. 53, 199–206.
Mengoni, F., Lichtner, M., Battinelli, L., Marzi, M., Mastroianni, C.M., Vullo, V., Mazzanti, G., 2002. In vitro anti-HIV activity of oleanolic acid on infected human mononuclear cells. Planta Med. 68, 111–114.
Morishima, C., Shuhart, M.C., Wang, C.C., Paschal, D.M., Apodaca, M.C., Liu, Y., Sloan, D.D., Graf, T.N., Oberlies, N.H., Lee, D.Y., Jerome, K.R., Polyak, S.J., 2010. Silymarin inhibits in vitro T-cell proliferation and cytokine production in hepatitis C virus infection. Gastroenterology 138, 671–681, 681.e671–672.
Pawlotsky, J.M., 2011. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. Hepatology 53, 1742–1751.
Pham, L.V., Ngo, H.T., Lim, Y.S., Hwang, S.B., 2012. Hepatitis C virus non-structural 5B protein interacts with cyclin A2 and regulates viral propagation. J. Hepatol. 57, 960–966.
Rai, R., Deval, J., 2011. New opportunities in anti-hepatitis C virus drug discovery: targeting NS4B. Antiviral Res. 90, 93–101.
Rehman, S., Ashfaq, U.A., Javed, T., 2011. Antiviral drugs against hepatitis C virus. Genet. Vaccines Ther. 9, 11.
Rice, C.M., 2011. New insights into HCV replication: potential antiviral targets. Top Antivir. Med. 19, 117–120.
Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.
Wang, M., Ng, K.K.-S., Cherney, M.M., Chan, L., Yannopoulos, C.G., Bedard, J., Morin, N., Nguyen-Ba, N., Alaoui-Ismaili, M.H., Bethell, R.C., James, M.N.G., 2003. Non-nucleoside analogue inhibitors bind to an allosteric site on HCV NS5B polymerase: crystal strucutres and mechanism of inhibition. J. Biol. Chem. 278, 9489–9495.
Waris, G., Sarker, S., Siddiqui, A., 2004. Two-step affinity purification of the hepatitis C virus ribonucleoprotein complex. RNA 10, 321–329.
Wu, Y., Liao, Q., Yang, R., Chen, X., 2011. A novel luciferase and GFP dual reporter virus for rapid and convenient evaluation of hepatitis C virus replication. Virus Res. 155, 406–414.
Ye, L., Timani, K.A., Kong, L., Yang, X., Liao, Q., Wu, J., 2005. Two cis-acting elements in negative RNA strand of Hepatitis C virus involved in synthesis of positive RNA strand in vitro. Acta Virol. 49, 83–90.
Zeuzem, S., 2008. Interferon-based therapy for chronic hepatitis C: current and future perspectives. Nat. Clin. Pract. Gastroenterol. Hepatol. 5, 610–622.
Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA 102, 9294–9299.