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Inhibition of hepatitis C virus replication by herbal extract: *Phyllanthus amarus* as potent natural source

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ABSTRACT

Hepatitis C virus infection is a major health problem worldwide. Developing effective antiviral therapy for HCV is the need of the hour. The viral enzymes NS3 protease and NS5B RNA dependent RNA polymerase are essential enzymes for polyprotein processing and viral RNA replication and thus can be potential targets for screening anti-HCV compounds. A large number of phytochemicals are present in plants, which are found to be promising antiviral agents. In this study, we have screened inhibitory effect of different plant extracts against the NS3 and NS5B enzymes of hepatitis C virus. Methanolic extracts were prepared from various plant materials and their inhibitory effects on the viral enzymes were determined by *in vitro* enzyme assays. Effect on viral RNA replication was investigated by using TaqMan Real time RT-PCR. Interestingly, *Phyllanthus amarus* root (PAR) extract showed significant inhibition of HCV-NS3 protease enzyme; whereas *P. amarus* leaf (PAL) extract showed considerable inhibition of NS5B in the *in vitro* assays. Further, the PAR and PAL extracts significantly inhibited replication of HCV monocistronic replicon RNA and HCV H77S viral RNA in HCV cell culture system. However, both PAR and PAL extracts did not show cytotoxicity in Huh7 cells in the MTT assay. Furthermore, addition of PAR together with IFN-α showed additive effect in the inhibition of HCV RNA replication. Results suggest the possible molecular basis of the inhibitory activity of PA extract against HCV which would help in optimization and subsequent development of specific antiviral agent using *P. amarus* as potent natural source.

1. Introduction

Hepatitis C virus (HCV) infection is one of the major health problems all around the world. Earlier reports suggest that patients with chronic infection of HCV have high risk of developing liver cirrhosis and often lead to hepatocellular carcinoma (Strader et al., 2004). The combination of pegylated interferon and ribavirin is the choice of treating HCV infection at present. This treatment has limited success rate of 40–50% sustained virological response and is associated with a wide range of side-effects including flu-like symptoms, anemia and depression leading to treatment discontinuation in a significant proportion of patients. HCV genome is a single-stranded RNA molecule of positive polarity, ~9600 nucleotides in length. HCV RNA encodes a polyprotein which is processed into at least 10 distinct products (structural proteins C, E1, and E2 and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by host and viral proteases (Bartenschlager, 2002). Among them, NS2 and NS3 protease are essential enzymes for polyprotein processing. Hence, they play an essential role in the replication of HCV (Lorenz et al., 2006; Welbourn and Pause, 2007; Schregel et al., 2009). Therefore, they are the potential targets for screening anti HCV compounds. The recent development of a subgenomic-replicon system in Huh7 cells and production of infectious HCV (H77S) particles provide a powerful tool for studying virus replication and for screening anti-HCV drugs (Zeisel and Baumert, 2006). Several inhibitors of NS3/4A protease and NS5B polymerase, either alone or in combination with Peg-IFNα/ribavirin, have shown encouraging results in clinical trials (Lenz et al., 2010).

A number of synthetic compounds are currently under clinical trial, which include Telaprevir (VX 950) and Boceprevir (SCH 503034) (Reesink et al., 2006; Sarrazin et al., 2007; Forestier et al., 2007; Chen et al., 2009). Natural products, especially plants have been used for the treatment of various diseases for thousands of years (Dholwani et al., 2008). Currently, research and development of new drugs from natural resources in a systematic and strategic manner has become the global trend. In the last decade and as an alternative to conventional chemical agents, a large number of phytochemicals have been recognized as a way to control infections

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caused by viruses. So far several plant extracts have been screened for their antiviral effect against many viral infections. Molecular studies on antiviral potentials of *Phyllanthus amarus* showed its inhibitory effect on HBV polymerase activity (Thyagarajan et al., 1988). Methanolic extracts of *Acacia nilotica*, *Boswellia carterii*, *Embelia schimperi*, *Quercus infectoria*, *Trachyspermum ammi* and aqueous extracts of *Piper cubeba*, *Quercus infectoria* and *Syzygium aromaticum* showed potentials to inhibit HCV (Hussein et al., 2000). In order to screen a natural potent inhibitor for HCV, we assayed methanolic extracts from ten different plants which are used in treating viral jaundice and liver tonics.

2. Materials and methods

2.1. Plasmid constructs

pYB-43 plasmid contains the single-chain protease NS4A-NS3 (scNS3) and pYB-44 vector contains NS3 cleavage site at middle flanked by Enhanced Green Fluorescent Protein (EGFP) and cellulose binding domain (CBD) (both are kind gift from Dr. Itai Benhar, Tel Aviv University, Israel). A cell culture adapted infectious H77s-cDNA construct (Yi et al., 2006) was kindly provided by Stanley Lemon, University of Texas, Galveston and was used to screen inhibitory effect of plant extracts against HCV replication.

The HCV replicon construct pSGRLuc JFHI (Generous gift from Dr. Takaji Wakita) (Kato et al., 2005) was linearized with Xba I and the HCV-Luc replicon RNA was generated using T7 RNA polymerase.

2.2. Preparation of plant extracts

Plants used in this study were identified and collected from their natural habitat and voucher specimens were stored in laboratory. Collected plants were shade dried and powdered. 50 g of each powder was extracted with 200 ml of methanol for 48 h at room temperature and filtered to remove insoluble debris. The extract was dried at low temperature with reduced pressure using rota evoparator (Buchi® Rotary Evaporators). During the assay, each extract was dissolved in sterile water with 0.2% dimethyl sulfoxide (DMSO, Sigma–Aldrich) into the final concentrations 1000 μg/ml.

Similarly, *P. amarus* root and leaf were separated, extracted with methanol, dried in rota-evaporator (Buchi® Rotary Evaporators) and dissolved in water.

2.3. HCV NS3 protease activity assay

The inhibitory effect of plant extracts against HCV NS3 protease was assessed earlier by high throughput fluorescent based assay. The cleavage of recombinant substrate (EGFP-NS5A/B site (SEDVVCCSMSY)-CBD) by recombinant HCV NS3 protease was performed as described earlier by Berdichevsky et al. (2003) with little modification. In brief 0.01–0.1 μM of recombinant protease and different dilutions of the plant extracts were pre-incubated in an eppendorf for 15 min at 37 °C in reaction buffer (50 mM Tris–HCl (Merck) pH 7.5, 150 mM NaCl (Merck), 0.05% Tween (Sigma–Aldrich), 20% Glycerol (Merck), 1 mM DTT (Sigma–Aldrich) 0.25–1.0 mM). The recombinant substrate was added to the above reaction mix and further incubated for 1 h at 37 °C, followed by the addition of cellulose slurry (20 mg cellulose (Sigma–Aldrich) pre-equilibrated with reaction buffer). The reaction tube was rocked for 10 min at 4 °C followed by centrifugation for 2 min at 12,000 rpm. The supernatant containing released EGFP was transferred to a black 96-well plate and fluorescence was measured using excitation filter 485 nm and emission filter 538 nm in a fluorometer (Modulus™ Microplate Multimode reader). The difference between the fluorescence obtained in the presence and absence of the extract was taken as the measure of the inhibitory activity. Results represent the average of three independent experiments.

2.4. NS3 protease assay ex vivo in Huh7 cells

Huh7 cells were first infected with vaccinia virus encoding T7 RNA Polymerase using standard protocol (Ray et al., 2006). This was followed by DNA transfection with constructs encoding NS3 protease enzyme (pYB-43) and the substrate (pYB-44) using Tfx-20 as the transfection reagent. Cells were then treated with PA extract (either 20 μg/ml or 40 μg/ml) and substrate level was detected by Western blot analysis by anti-GFP antibody after 24 h of extract treatment.

2.5. NS5B assay for the measurement of inhibitory effect of extract

The NS5B gene of HCV was inserted into Nde1/Not1 restriction sites of pET29a expression vector to obtain recombinant NS5B/pET29a clone. NS5B enzyme was over expressed in *Escherichia coli* BL21 (DE3) cells and purified using published protocol. The HCV-NS5B activity was measured using radiolabeled α-P^{32} UTP in 30 μl assay buffer which includes 20 mM Tris (Merck) (pH 7.0), 100 mM NaCl (Merck), 0.5 mM DTT (Sigma–Aldrich), 0.01% Tween-20 (Sigma–Aldrich), 5% glycerol (Merck), and 0.5 mM $MnCl_2$ (Merck). In this assay, the enzymatic reaction mixture comprising of NS5B and polyA-dT were incubated on ice for 5 min. Reactions were started by addition of radiolabeled UTP mixture (BRIT) and were incubated for 1 h at 30 °C. The reactions were terminated by the addition of ice-cold 5% (v/v) trichloroacetic acid (Sigma–Aldrich) (TCA) containing 0.5 mM sodium pyrophosphate (Sigma–Aldrich) and were left overnight for precipitation at 4 °C. The quenched reaction mixtures were then transferred to GF-C filters, washed with chilled 5% TCA buffer to remove unincorporated UTP followed by washing with water and ethanol before vacuum drying. The amount of radioactive UMP incorporated into RNA products was quantified on a LKB wallac 1209 Rack Beta liquid scintillation counter. To measure the inhibitory effect of extracts on HCV NS5B polymerization, extract dilutions were made in DMSO (Sigma–Aldrich) and different concentration of inhibitors were added to NS5B enzyme reaction mixture. Negative control consisted of 10% DMSO (Sigma–Aldrich) along with enzyme and activity was measured as mentioned above. Results represent the average of three independent experiments.

2.6. MTT assay to measure the toxicity of extract

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used for the screening of extract's toxicity using standard protocol (Loske et al., 1998). Briefly, Huh7 cells were plated for 24 h at 37 °C on 96-well plates at a density of 5×10^3 cells per well to 50% confluency, with 5% CO_2 in a humidified atmosphere with Dulbecco's modified Eagle's medium. After 24 h, extracts were added to final concentrations of 10, 20, 40 and 80 μg/ml. The plate was incubated for further 78 h under the same conditions mentioned above. 20 μl MTT (Sigma–Aldrich) solution (5 mg/ml in phosphate buffer) was added to each well and incubated at 37 °C for 4 h. The MTT solution was carefully decanted off, and formazan was extracted from the cells with 100 μl of DMSO in each well. Color was measured with a 96-well ELISA plate reader at 550 nm, with the reference filter set to 620 nm. All MTT assays were repeated three times.

2.7. In vitro HCV replication inhibition assay in replicon 2a cells

Huh7 cells harboring HCV monocistronic replicon genotype 2a (Frese et al., 2003), a kind gift from Dr. Ralf Bartenschlager,