An Extracted Fraction of *Pseudomonas Oleovorans* Can Inhibit Viral Entry and RNA Replication of Hepatitis C Virus in Cell Culture

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**ABSTRACT**

The emergence and distribution of Hepatitis C virus (HCV) infection is still considered as an unsolved problem. Due to side effects, many synthetic drugs have been avoided and replaced by new biologically derived ones. Aim of this study was to use *Pseudomonas oleovorans*’ extract as HCV viral replication inhibition agent in cell culture system. Several factors were studied and the optimum growth conditions were selected for maximum production of antiviral substance. *Pseudomonas oleovorans*’ extract was fractionated using different concentrations of chloroform: methanol on silica gel columns. Analysis of potent fraction by GC/MS showed tetradecanoic and hexadecanoic acid methyl esters. The selected fraction was tested against HCV in vitro using two different protocols: viral attachment entry inhibition (Pre-incubation) and viral replication inhibition (Post infection). 0.1 µg / ml of the selected antiviral fraction resulted in inhibition of viral replication in Huh 7.5 cells. However, higher concentration of 100 µg / ml did not cause any viral inhibition. The selected bacterial fraction containing tetradecanoic acid and hexadecanoic acid methyl esters could be used as a promising candidate to inhibit viral HCV entry and replication of HCV.

**Key words:** Pseudomonas oleovorans, Replication, Viral inhibition, HCV and Antiviral

**Introduction**

Treatment of viral infections by biological methods could be better than chemical medicines due to its wide availability and fewer side effects. The biological activity of some synthetic peptides includes antifungal, antibacterial, anti-parasitic, antiviral and antitumor activities ¹². Hepatitis C virus (HCV) represents one of the main causes of chronic liver diseases leading to hepatic steatosis, fibrosis, cirrhosis and hepatocellular carcinoma. There is no vaccine available to prevent HCV infection due to the high mutation rate during replication among different genotypes. HCV standard of care until relatively recently was a combination of pegylated interferon (peg/IFN) and ribavirin, and it was only efficient in half of treated patients, with many adverse effects. For instance, in patients with malignant midgut carcinoid tumors treated with rhIFN-α2a, 41% developed anti-IFN-α antibodies and 32–42% in patients with metastatic renal cell carcinoma. Moreover, it was reported that PEG-IFN-α preparations with longer half-life are immunogenic when given to HCV patients previously receiving conventional IFN-α therapy³. However, new interferon-free combinations of direct acting antivirals (DAAs) have revolutionized HCV treatment with better response and fewer side effects. Treatment of HCV has rapidly progressed with the DAAs development targeting genome replication and/or polyprotein processing. DAAs target virally encoded enzymes through a poorly defined mode of action. While the success of these therapies has been encouraging to date, the long-term response and potential for viral resistance remain to be seen⁴. Several HCV cell culture systems as sub genomic replicons, retroviral HCV pseudo particles (HCVpp) and the JFH1-based infection system have exposed more information about the molecular mechanisms involved in
HCV replication. Infectious cell culture systems recap the entire viral life cycle allowing the studying of the effect of all DAAs and HTAs (Host targeting agents). Working with primary cells and polarized cells has recently enhanced which may help to address some limitations of the previously established cell lines. Bovine virus diarrhea virus (BVDV) is a culture model virus for screening compounds with anti-HCV neutralizing activity due to the close relation between the two viruses in sharing significant degree of local protein homology and a common replication strategy.

HCV infection as a major cause of chronic liver disease extends the need for HCV effective prophylactic vaccine globally. Research is extended in using HCV cell culture system to identify the essential host factors to complete the life cycle of HCV and to identify the mechanisms of various interactions between HCV and the infected host. A novel production system for vaccine against HCV was established using Vero cells. The presence of natural antiviral agents against HCV has been developed during the last few years. Aqueous enzymatic extracts of edible mushroom, Agaricus bisporus (AhAEE), have been studied as a key source of biological active compounds against HCV proteases. Grape seed extract (GSE) containing more amounts of flavonoids exerted inhibitory effects on HCV replication without causing host cellular toxicity. Soraphen A (SorA) is a myxobacterial metabolite with a very potent anti-HCV activity. Echinocystic acid, an oleanane-type triterpene, displayed substantial inhibitory activity on HCV entry. Also, a lipophilic long-chain compound derived from microbial metabolites, an inhibitor of sphingolipid biosynthesis, was shown to inhibit HCV replication.

In this study, microbial extract derived from Pseudomonas oleovorans was used as a potentially inhibitory agent of HCV viral replication in cell culture system. The fractions were extracted with different concentrations of chloroform: methanol on silica gel columns by column fraction. The selected fraction was tested against HCV in vitro with two different protocols, to assess inhibition of viral attachment and entry (Pre-incubation) and viral replication (Post infection), respectively.

Materials and Methods

Purification and testing of antiviral substance from the culture supernatant of Pseudomonas oleovorans was carried out in our previous study by extraction with ethyl acetate. The ethyl acetate crude extract was fractionated by methanol (90%) and n-hexane. The methanol fraction was further fractionated to detect and identify the potent antiviral compound against BVDV.

Gas Chromatography/ Mass Spectrometry analyses

Agilent 6890 gas chromatography equipped with an agilent mass spectrometric detector with a direct capillary interface and fused silica capillary column PAS-ms (30m×0.25mm I.D.×0.25µm film thickness) was used as described earlier. Helium was used as carrier gas at approximately 1ml/min pulsed split less mode to inject samples. The solvent delay was 3 min and the injection size was 1.0 µl. The mass spectrophotometric detector was operated in electron impact ionization mode an ionizing energy of 70e.v. scanning from m/z 50 to 550. The ion source temperature was 250°C and the quadrupole temperature was 150°C. The electron multiplier voltage (EM voltage) was maintained 1550 v above auto tune. The instrument was manually tuned using perfluorotributyl amine (PFTBA). The GC temperature program started at 60°C then was elevated to 280°C at the rate of 8°C/ min and15 min holding at 280°C, the injector temperature was set at 280°C. Wiley mass spectral data base was used in the identification of the separated peaks.

Establishment of cell culture system infected with HCV

Huh7, a well differentiated human hepatocellular carcinoma cell line modified to produce a more efficient Huh7.5 cell line was a generous gift from Charles Rice (The Rockefeller University, USA) obtained under Material Transfer Agreement (MTA# 642 to Dr. Mostafa K. El Awady 2007). HCV infection experiments and assessment of viral replication were done on Huh7.5 cells. HCV positive serum was collected from HCV infected patient examined at the Medical Unit at the National Research Centre, Cairo, Egypt. The diagnosis of this patient was based on physical examination and serological testing, for the presence of anti-HCV-antibodies in the serum, using a recombinant HCV antigen based test (Axium HCV Rapid test, Florida, U.S.A.). The HCV infection was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) as mentioned in our previous studies.

Huh7.5 cells were cultured and infected with HCV particles according to protocols described by Seipp et al. and Song et al. Successful viral infection in Huh7.5 cells throughout the culture duration was confirmed by Reverse Transcription Polymerase Chain Reaction (RT-PCR) amplification of HCV RNA at the transcriptional level. Retro-transcription was performed in 25 µl reaction mixture containing 3 µl (~400 ng) of the extracted RNA, a final concentration of 0.2 mM from each dNTP (MBI Fermentas; Germany) and 50 pmol of primers 1CH or 2CH for transcriptional level.

The primers was as following: 1CH: 5'-GGTGCACGGTCTCA-GAGACCTC-3' and 2CH: 5'-AACTACTGTCTCTCAGCAGAA-3'. A total of 40U RNAs in (Clontech; U.S.A.), 20U of reverse transcriptase (RT) isolated from Avian Myeloblastosis Virus and 2.5 µl 10X enzyme buffer (AMV RT; Q-BIO gene, Germany) were added to the mixture, the reaction was incubated in the thermal cycler (T1; Whatman Biometra, Germany) at 42°C for 60 min, then the RT was inactivated and double stranded cDNA was denatured at 95°C for 10 min and chilled on ice to keep the DNA strands apart. The amplification
All tubes were incubated at 37°C for three hours.

of 1 ml FCS-free DMEM. Infectious and healthy (HCV sample was added to dilutions of the selected fraction RT-PCR. Briefly, in a 1.5 ml tube, HCV infectious serum cells. HCV binding to target cells was detected by nested viral load 1.136.648 IU/ml prior to application to Huh7.5 fraction were pre-incubated with HCV serum sample of infected cells, different concentrations of the selected entry inhibition assay (Pre-incubation)

1 mg/ml concentration was considered as toxic signs of toxicity, e.g. partial or complete loss of monolayers. Huh7.5 cells were observed for any physical concentrations in comparisons with non-treated cells discussed before in our previous study using different concentrations from the selected fraction used in both pro and 10 µg/ml as reported in our previous study was examined at concentrations of 1mg/ml, 100 µg/ml, 0.1 µg / ml and 0.001M EDTA, pH 8.0, as reported by El Abd et al., Evaluation of the antiviral activity of the selected bacterial fraction using viral replication inhibition assay (Post infection) Huh7.5 cells (200,000 cells / well) were grown to semi-confluence for 48 h in 5 % CO₂ incubator. The media were aspirated, then cells were washed twice with FCS-free medium and inoculated with 200 µl HCV sera diluted with FCS-free DMEM and after 90 minutes of incubation, 1.8 ml DMEM containing 10% FCS was added to each well. Two wells were left without sera inoculation (supplied just with DMEM) to serve as negative controls. The plates were incubated for 24 h. After incubation media were aspirated and cells were washed three times with PBS to get rid of any remaining viral particles. Then 500 µl of the previously prepared dilutions of the selected fraction concentrations 100 µg / ml, 0.1 µg / ml in a final volume of 1ml FCS-free DMEM were added. Plates were incubated at 37°C in 5% CO₂. On the next day, media were aspirated and 500 µl of solution D was added to each well and left for 5 min or until the cells were completely lysed by the solution and then the cell lysate was transferred to a 1.5 ml tube. RNA extraction was performed from the cell lysate using acid guanidium-phenol-chloroform method, then HCV RNA detection was performed qualitatively by nested RT-PCR and the amplicons were visualized using agarose gel electrophoresis as reported in our previous study Evaluation of the antiviral activity of the selected bacterial fraction using viral attachment entry inhibition assay (Pre-incubation) (Tabil et al., and Yasuhara-Bell et al.,)

In order to study the effect of the selected bacterial fraction from Pseudomonas oleovorans extract on HCV infected cells, different concentrations of the selected fraction were pre-incubated with HCV serum sample of viral load 1.136.648 IU/ml prior to application to Huh7.5 cells. HCV binding to target cells was detected by nested RT-PCR. Briefly, in a 1.5 ml tube, HCV infectious serum sample was added to dilutions of the selected fraction concentrations 100 µg / ml, 0.1 µg / ml in a final volume of 1 ml FCS-free DMEM. Infectious and healthy (HCV negative) sera diluted 1:50 in 1 ml FCS-free DMEM were included as positive and negative controls, respectively. All tubes were incubated at 37°C for three hours.

Huh7.5 cells (200,000 cells / well) were grown to semi-confluence in DMEM supplied with 10% FCS, 1% Penicillin-Streptomycin and 0.1% fungizone in 6 well plates for 48 h. After media were removed, cells were washed with FCS-free medium twice and inoculated with 1ml of the previously incubated sera-fraction mix. For each assay, a negative control consisted of two wells supplied with DMEM without sera inoculation. After 90 minutes at 37°C in 5% CO₂, 1ml DMEM containing 10% FCS was added to each well. On the second day, media were aspirated and to get rid of any remaining sera-fraction mix cells were washed three times with PBS, replaced with fresh medium DMEM without FCS and cells were further incubated for 24 hrs at 37°C. On the third day, media were aspirated and 500 µl of solution D was added to each well and left for 5 min or until the cells were completely lysed by the solution and then the cell lysate was transferred to a 1.5 ml tube. RNA extraction was performed from the cell lysate using acid guanidium-phenol-chloroform method, then HCV RNA detection was performed qualitatively by nested RT-PCR and the amplicons were visualized using agarose gel electrophoresis as reported in our previous study Results

The GC/MS analysis of the fraction with antiviral activity against BVDV revealed the presence of saturated fatty acids methyl esters (presence of tetradecanoic acid and hexadecanoic acid methyl esters) as shown in figure 1.nAbundance of Hexadecanoic acid methyl ester C16H34O2 at retention time 18.97 min. and Tetradecanoic acid 12-methylester C15H30O2 at 17.33 min.
The nested RT-PCR products (174 bp) were visualized on 2% agarose gels. As shown in figures 2 and 3, *Pseudomonas oleovorans* extract with the concentration of 100 µg/ml cannot prevent viral attachment inhibition (pre-infection) or HCV viral replication inhibition (post infection). While the extracted fraction of *Pseudomonas oleovorans* with the concentration 0.1µg/ml inhibited viral entry and RNA replication of hepatitis C virus in cell culture as shown in figures 2 and 3.

**Discussion**

In the present study, evaluation of the selected bacterial fraction for antiviral activity against HCV has been detected using both inhibition viral entry (pre-infection) and RNA replication inhibition assay (post infection). Results of the first inhibition assay indicated that, 100 µg/ml from the selected fraction did not affect viral attachment, however, the use of 0.1 µg/ml of the fraction led to full inhibition of the virus. This indicated the probability of using this fraction to block both viral attachment and entry to the host cell. As, it was able to neutralize the virus infectivity through binding to and/or blocking the virus itself or through blocking cellular receptors which are responsible for the entry of the virus in the host cell as shown by RT-PCR for HCV RNA.

The second method for determination of antiviral activity against HCV was through the viral replication inhibition assay. In this method cells were infected with HCV RNA positive serum before adding the selected fraction of the bacterial extract. Treating the infected HCV cells with two different concentrations of the selected fraction led to inhibition of viral replication using lower concentration rather than the high one. When the fraction was added to the infected cells the virus lost its ability to re-infect new host cells. It is not yet known if this is due to the inhibitory effect of the fraction of the bacterial extract against the active molecules of the virus or due to the decreased infectivity of the virus released substances in to the media. The same explanation was suggested for the antiviral activity of extracted marine microorganisms against herpes simplex virus (HSV-1) and vesicular stomatitis virus (VSV). Several polyunsaturated fatty acids (PUFAs) as eicosapentaenoic acid (EPA), arachidonic acid (AA) and docosahexaenoic acid (DHA) showed anti-HCV activities using HCV sub genomic RNA replicon system. The mechanism of action PUFAs in the inhibition of HCV replication was not clear. Consistent with the importance of the fatty-acid-biosynthetic pathway in HCV RNA replication, saturated and monounsaturated fatty acids can improve RNA
replication of HCV. HCV replication has been demonstrated to occur in an HCV-induced “membranous web” consisting of modified lipid-containing intracellular vesicles; it is possible to suggest that fatty acids are required for the right folding of this structure that is required for HCV RNA replication. Inhibition of fatty acid synthase (FASN) led to a decrease in the replication of sub genicomic HCV replicons as well as HCV virion production. Although the underlying mechanisms are not yet completely understood, these studies imply that FASN was crucial for the virus replication. This contributed to studying the use of fatty acids for inhibition of HCV as a source for new drug development. Viral resistance has evolved with the development of several anti-HCV drugs, thus new drugs inhibiting the host factor that contributes to HCV replication in arrangement with the era of the DAAAs may solve this problem. The main element in the efficacy of viral replication is well-defined through an enzyme for long-chain mono-unsaturated fatty acid (LCMUF) synthesis called stearyl-CoA desaturase (SCD). SCD-1 inhibitors could be used together with interferon-alpha as multi-drug anti-viral agents against HCV. Since the advent of (DAA) agents, chronic HCV treatment has evolved at a rapid pace. In contrast to prior regimens involving ribavirin and pegylated interferon, these newer agents are highly effective, well-tolerated, have shorter course of therapy and are safer essentially in all HCV patients including those with advanced liver disease and following liver transplantation.

A similar study on _Pseudomonas oleovorans_ strain KBPF-004 (developmental code KNF2016) showed antiviral activity against mechanical transmission of toboviruses. Antiviral activity was also evaluated in seed transmission of two toboviruses, _Pepper mild mottle virus_ (PMoV) and _Cucumber green mosaic virus_ (CGMV), by treatment of seeds collected from infected pepper and watermelon, respectively. Supernatant of _P. oleovorans_ strain KBPF-004remodelled aggregation of PMoV 12 kDa protein and subcellular localization of movement protein in _Nicotiana benthamiana_, diminishing aggregation of the 126 kDa protein and essentially abolishing association of the movement protein with the microtubule network. In leaves agro infiltrated with constructs expressing the coat protein (CP) of either PMoV or CGMV, less full-size CP was detected in the presence of supernatant of _P. oleovorans_ strain KBPF-004. These changes may contribute to the antiviral effects of _P. oleovorans_ strain KBPF-004. Further study could use a new strategy based on substitution of human serum infected Huh7.5 cells with HCVcc infection system, and then apply treatment with purified compounds extracted from bacterial isolates. This may contribute in part to the inference with HCV replicating system. In addition, preclinical studies in small experimental animals to study the cytotoxicity of bacterial extracts (liver and kidney functions, hematological and pathological changes) should be carried out. This might give new venues to be applied for the treatment of HCV.

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**References**

E. K. EXTIRIRANA FRAKCIJA PSEUDOMONAS OLEOVORANS MOŽE INHIBIRATI ULAZAK VIRUSA I REPLIKACIJU RNA VIRUSA HEPATITISA C U KULTURI STANICA

SAŽETAK

Pojava i distribucija infekcije virusom hepatitisa C (HCV) i dalje se smatra neriješenim problemom. Zbog nuspojava, mnogi sintetički lijekovi se izbjegavaju i zamijenju novim biološki dobivenim lijekovima. Cilj ovog istraživanja bio je koristiti ekstrakt Pseudomonas oleovorans kao sredstvo za inhibiciju replikacije HCV virusa u sustavu stanične kulture. Proučavano je nekoliko čimbenika i odabrani su optimalni uvjeti rasta za maksimalnu proizvodnju antivirusan tvari. Ekstrakt Pseudomonas oleovorans je frakcioniran korištenjem različitih koncentracija kloroform: metanol na stupcima silikagela. Analiza jake frakcije pomoću GC / MS pokazala je metil estere tetradekanoične i heksadekanske kiseline. Odabrana frakcija je testirana na HCV in vitro pomoću dva različita protokola: inhibicija ulaska virusa (pre-inkubacija) i inhibicija replikacije virusa (Post infekcija). 0,1 ug / ml odabrane antivirusan frakcije rezultiralo je inhibicijom virusne replikacije u Huh 7,5 stanicama. Međutim, veća koncentracija od 100 ug / ml nije uzrokovala nikakvu virusnu inhibiciju. Odabrana bakterijska frakcija koja sadrži tetradekanoičnu kiselinu i metil estere heksadekanske kiseline može se smatrati kandidatom za inhibiciju virusnog HCV ulaska i replikacije HCV.