Pan-genotypic hepatitis C virus inhibition by natural products derived from the wild Egyptian artichoke

Running Title: Compounds of wild Egyptian artichoke inhibit HCV.

Mahmoud Fahmi Elsebai [a,b,c,d ☯], George Koutsoudakis[e* ☯], Verónica Saludes[e,$], Gemma Pérez-Vilaró[e], Ari Turpeinen[b], Sampo Mattila[b], Anna Maria Pirttilä[d], Fabien Fontaine-Vive[c], Mohamed Mehiri[c], Andreas Meyerhans[f,g], and Juana Diez[e#]

[a]Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Egypt.

[b]Department of Chemistry, University of Oulu, PO Box 3000, FIN-90014 Oulu, Finland.

[c]Nice Institute of Chemistry, UMR CNRS 7272, UFR of Sciences, University Nice Sophia Antipolis, France.

[d]Department of Biology, University of Oulu, PO Box 3000, FIN-90014 Oulu, Finland

[e]Molecular Virology Group, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

[f]Infection Biology Group, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

[g]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain.

[*]present address: Liver Unit, Hospital Clinic, Institut D´Investigacions Biomèdics August Pi i Sunyer (IDIBAPS), Barcelona Spain.

[$]present address: Microbiology Service, Germans Trias i Pujol University Hospital, Germans Trias i Pujol Health Sciences Research Institute (IGTP), Badalona, Spain; and Centro de Investigación Biomédica en Red (CIBER) en Epidemiología y Salud Pública (CIBERESP), Instituto de Salud Carlos III, Madrid, Spain.

[☯] These authors contributed equally to this work

[#]Correspondence should be addressed to:

Juana Díez, PhD

Molecular Virology Group

Department of Experimental and Health Sciences

Universitat Pompeu Fabra

Doctor Aiguader 88, 08003, Barcelona, Spain

Tel: 00-34-933160862; Fax: 00-34-933160901

e-mail: juana.diez@upf.edu

ABSTRACT

Hepatitis C virus (HCV) infection is the leading cause of chronic liver diseases. Water extracts of the leaves of the wild Egyptian artichoke (WEA) (*Cynara cardunculus* L. var. *sylvestris* (Lam.) Fiori) have been used for centuries in Sinai Peninsula to treat hepatitis symptoms. Here, we isolated and characterized six compounds from the water extract of WEA and evaluated their HCV inhibition capacity *in vitro*. Importantly, two of these compounds namely grosheimol and cynaropicrin inhibited HCV with half maximal effective concentrations (EC_{50}) in the low micromolar range. They inhibited HCV entry into target cells and were active both against cell-free infection as well as cell-cell transmission. Furthermore, the antiviral activity of both compounds was pan-genotypic as HCV genotypes 1a, 1b, 2b, 3a, 4a, 5a, 6a and 7a were inhibited. Thus, grosheimol and cynaropicrin are promising candidates for the development of new pan-genotypic entry inhibitors of HCV infection.

IMPORTANCE

Because there is no preventive HCV vaccine available today, the discovery of novel anti-HCV cell entry inhibitors could help develop preventive measures of infection. The present study describes two compounds, isolated from the wild Egyptian artichoke (WEA), with respect to their structural elucidation, absolute configuration and quantitative determination. Importantly, both compounds inhibited HCV infection *in vitro*. The first compound was an unknown molecule and it was designated grosheimol while the second compound is the known molecule cynaropicrin. Both compounds belong to the group of sesquiterpene lactones. The mode-of-action of these compounds was during the early steps of the HCV life cycle, including cell-free and cell-cell infection inhibition. These natural compounds present promising candidates for further development into anti-HCV therapeutics.

INTRODUCTION

The hepatitis C virus (HCV) is an enveloped, positive strand RNA virus classified as a separate genus (Hepacivirus) within the Flaviviridae family. It shows a high degree of genetic diversity with 7 major circulating genotypes (1). HCV is mainly transmitted through exposure to HCV-contaminated blood. Most infections remain persistent summing up to an estimated 150 million chronic HCV carriers worldwide (2). As persistent HCV infection frequently causes chronic hepatitis that can progress to liver cirrhosis and liver cell carcinoma, it is a major threat for human health (3, 4).

Treatment options for chronically infected individuals have dramatically improved over the last years. This was due to the development of highly potent direct-acting antivirals (DAAs) that increased sustained response rates even in interferon-free combinations to over 90% (5). Currently, approved DAAs include NS3/4A protease inhibitors (telaprevir, boceprevir and simeprevir), NS5A inhibitors (daclatasvir and ledipasvir) and the NS5B polymerase inhibitor sofosbuvir. Further antiviral drugs are in clinical trials and about to be approved. Nonetheless, the rapid replication of HCV, along with the error-prone NS5B polymerase that lacks proof-reading activity, give rise to the generation of mutations throughout the viral genome. This results in remarkable sequence variation of an infecting HCV population, also known as a quasispecies. Amongst an HCV quasispecies drug resistant mutants are likely to be present and able to spread between individuals (6, 7). Furthermore, the current treatments are extremely expensive and thus present a significant burden for healthcare systems (8). Therefore, there is a continued interest in developing further antiviral drugs with low production costs. As there is no

preventive vaccine against HCV infection, another major interest is the development of effective preventive measures of infection.

Artichokes are part of the Mediterranean diet and rich in pharmaceutically active compounds like caffeoylquinic acid derivatives, sesquiterpene lactones and flavonoids (9). They are used since ancient times in folk medicine especially against liver complaints. For example, Bedouins in Sinai Peninsula (the Asian part of Egypt) use water extracts of the leaves of the wild Egyptian artichoke (WEA) (*Cynara cardunculus* L. var. *sylvestris* (Lam.) Fiori) to treat diverse symptoms of hepatitis like jaundice and ascites. Based on this traditional use we aimed to explore whether the WEA leaf extract may contain compounds with anti-HCV activities. Here we describe the isolation, of 6 natural products from water extracts of the WEA. Two of the 6 compounds, namely cynaropicrin and grosheimol, showed potent antiviral activities against all HCV genotypes and therefore were characterized extensively with respect to their mode-of-action. Our studies reveal that both compounds interfere with HCV infection of target cells at an early step. Thus, grosheimol and cynaropicrin represent interesting hit compounds for further development of cost-effective anti-HCV drugs.

MATERIALS AND METHODS

Cell culture

Huh7/Scr cells, Huh7.5.1 Cl.2 cells (kindly provided by F. Chisari, The Scripps Research Institute, La Jolla, CA), and 293T cells (HEK293T cells, American Type Culture Collection, Manassas, VA, CRL-1573) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 units/ml penicillin and 100 units/ml streptomycin (DMEM complete). Huh7.5/EGFP-NLS-IPS stable cell lines were generated by plasmid transfection of a commercial pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA) expressing the enhanced green fluorescent protein (EGFP) followed by nuclear localization signal (NLS, PKKKRKVG) and (IFN)-β promoter stimulator protein 1 (IPS-1) and subsequent selection with G418 as previously described (10). Cells were grown in an incubator with 5% CO_2 at 37ºC.

Plasmids

The plasmid pFK-Jc1 has been previously described (11). The subgenomic replicon plasmid pSGR-JFH1 carries a bicistronic construct where a firefly luciferase gene is expressed via the HCV IRES and an EMCV IRES drives expression of JFH1 non-structural proteins (NS3 to NS5B) (12). pTN7-Stopp is a HIV plasmid that carries the renilla luciferase reporter gene instead of the nef gene and lacks a functional env gene, thus set to produce only a single-round of infection (13). The plasmid which encodes E1E2 glycoproteins of the strain HC-J6CH, named pcDNA3.1-ΔcE1E2-J6CH, has been described elsewhere (14). The plasmid pVPack-VSV-G

which encodes the vesicular stomatitis virus glycoprotein (VSV-G), was purchaced by Agilent Technologies (Santa Clara, CA). HCV genotype 1-7 plasmids are JFH-1 based reporter virus constructs, carrying renilla luciferase inserted at the NS5A gene and structural proteins from all major HCV genotypes (15).

Control compounds for HCV inhibition experiments

The 2'-modified nucleoside analog (2'-C-methyladenosine abbreviated 2'-C-Met) was kindly provided by Dr. Pablo Gastaminza (Spanish National Biotechnology Center, Madrid, Spain). Dasatinib, erlotinib and U0126 were purchased from Selleck Chemicals (Houston, TX). For compounds stock preparation, each individual compound was diluted in DMSO at a final concentration of 10 mM. Chlorpromazine and heparin sodium salt were purchased from Sigma-Aldrich (St. Louis, MO) and stock solutions were prepared in H_20 at a final concentration of 50 mg/ml and 100 mg/ml, respectively.

In vitro transcription and electroporation of HCV RNA

The plasmid carrying the Jc1 construct was linearized with the MluI enzyme while the pSGR-JFH1 was linearized with the XbaI enzyme. Linearized plasmid DNA was purified with the QIAquick PCR purification kit (Qiagen, Düsseldorf, Germany). Purified DNA was subjected to an in vitro transcription reaction with the MEGAscript® T7 kit (Applied Biosystems, Foster city, CA) according to the manufacturer's protocol. RNA from the in vitro transcription reaction was purified with the Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany), RNA integrity was verified by formaldehyde agarose gel electrophoresis and the concentration was determined

by measurement of the optical density at 260 nm. For RNA electroporations, single cell suspensions of Huh7.5.1 Cl.2 cells were prepared by trypsinization of cell monolayers. Cells were washed with phosphate-buffered saline (PBS), counted, and resuspended at 1.5×10^7 cells per ml in Cytomix (16) containing 2mM ATP and 5mM glutathione. Ten µg of in vitro transcribed RNA was mixed with 400 µl of the cell suspension. Cells were then electroporated, immediately transferred to 10 ml of culture medium and seeded in a 10-cm dish. Electroporation conditions were 975 µF and 270 V by using a Gene Pulser Xcell™ system (Bio-Rad, Munich, Germany) and a cuvette with a gap width of 0.4 cm (Bio-Rad).

Generation of HCVcc stocks and determination of virus titers in cell culture supernatants.

For the generation of HCVcc stocks, supernatants of the electroporated cells were harvested 72 h post electroporation, cleared by passing them through 45-µm-pore-size filters and stored at -80ºC. For the determination of viral titers Huh7/Scr cells were seeded at a concentration of 1.2×10^4 cells per well in a 96-well plate in a total volume of 200 µl. Twenty-four hours later, serial dilutions of virus containing supernatant were added (6 wells per dilution.) Three days later, cells were washed with PBS and fixed for 20 min with ice-cold methanol at −20°C. After three washes with PBS, NS5A was detected with a 1:2000 dilution of the α-NS5A antibody 9E10 (kindly provided by C. Rice, The Rockefeller University, NY) in PBS supplemented with 5% BSA for 1 h at room temperature. Cells were washed again three times with PBS and bound primary antibodies were detected by incubation in PBS-5% BSA with goat α-mouse IgG-peroxidase conjugated antibody (Sigma-Aldrich, St. Louis, MO) at 1:400 dilution. After 1 h incubation at room temperature, cells were washed three times with PBS; the Vector NovaRED substrate kit (Linaris Biologische Produkte GmbH, Wertheim, Germany) was used for detection

of peroxidase. Virus titers [50% tissue culture infective dose per ml ($TCID_{50}$/ml)] were calculated based on the method described by Spearman and Kärber.

Luciferase assays and cell cytotoxicity (viability) assays

For standard infection assays Huh7/Scr cells were seeded at a density of 1.2 x 10^4 cells/well in 96-well plates. One day later cells were pre-incubated for 1h at 37ºC with the pertinent compounds and then inoculated with the virus and the compounds for 4h at 37ºC. HCVpp were left for 6h. Finally, virus-containing media was replaced by a fresh media-compounds mix. Firefly and renilla luciferase activities were assayed 72h post infection with the Dual-Glo® Luciferase Assay System while cytotoxicity (viability) assays were carried out with the CytoTox-Glo™ Cytotoxicity Assay (both purchased from Promega Corporation, Madison, WI), according to manufacturer's instructions, using a plate luminometer FLUOstar OPTIMA (BMG LABTECH, Ortenberg, Germany). Mean relative light units (RLU) were plotted as percentage relative to control infections (solvent without compounds) for both infectivity and cell viability. Infections were carried out in duplicates and measured in duplicates (mean ± SEM; n=4). Half maximal effective concentration (EC_{50}) and half maximal cytotoxic concentration (CC_{50}) were estimated by non-linear regression of log inhibitor vs. normalized response and used to calculate the Selectivity Index (SI) value.

Preparation of HCV pseudoparticles (HCVpp)

HIV-based pseudoparticles bearing HCV glycoproteins were generated by calcium phosphate cotransfection of 293T cells. Briefly, 3.6 x 10^6 293T cells were seeded in 10-cm dishes one day

before transfection with equal amounts of pTN7-Stopp and pcDNA3.1-ΔcE1E2-J6CH or pVPack-VSV-G plasmids. A total amount of 20 µg of DNA was mixed with a 2M $CaCl_2$ solution and then, 2X Hepes-buffered saline (HBS) was added dropwise to form a precipitate which was added to the cells. The medium was replaced on the following day and supernatants containing the pseudoparticles were harvested 48 h later, cleared by passage through 0.45-µm-pore-size filters, and used for luciferase infection assays.

Subgenomic replicon assay

Huh7/Scr cells were seeded at a density of 5 x 10^4 cells/well in 24-well plates. On the following day, cells were transfected with electroporation with RNA of the SGR-JFH1 according to the protocol described above. Four hours post transfection, transfection medium was replaced by fresh medium and compounds were added. Firefly luciferase activity was measured 48h later as described above.

Cell-cell transmission assay

Huh7/Scr cells ("donor cells") were infected with the Jc1 virus at an MOI ≥5 $TCID_{50}$/cell. 24h later cells were trypsinized, counted and 5 x 10^4 cells were co-cultured in 24 well plates on cover slips with 5 x 10^4 Huh7.5/EGFP-NLS-IPS cells ("acceptor cells"). Cynaropicrin (10 µg/ml), grosheimol (20 µg/ml), erlotinib (10 µg/ml) or DMSO (0.1 % v/v) were added simultaneously to the cells during seeding. Four hours later, medium was removed, cells were washed once with PBS and finally covered with fresh DMEM complete, containing 1% low melting agarose (Sigma-Aldrich, St. Louis, MO) and cultivated for another 20 h prior to immunofluorescence

analysis. To assess the efficiency of cell-cell infection, the proportion of infected cells was monitored by NS5A-specific indirect immunofluorescence (as described below). Quantification of Huh7.5/EGFP-NLS-IPS cells was done in 3 independent wells by taking 3 independent pictures of different field of each well that contained at least 200 cells in total. Data are expressed as percentage to DMSO treated cells and represent mean values of the 3 independent fields of three biological replicates (±SEM).

Indirect immunofluorescence

Twenty four h post co-cultivation of Huh7/Scr with Huh7.5/EGFP-NLS-IPS wells, medium containing 1% agarose was aspirated after heating at 42 ^{o}C for 10 min. Then cells were washed 3x with PBS, fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS. Immunostaining of NS5A was performed by using the mouse monoclonal α-NS5A antibody 9E-10 (kindly provided by Prof. Charles Rice, The Rockefeller University, USA) at a final concentration of 0.1 µg/mL in PBS supplemented with 5% bovine serum albumin (BSA). Bound primary antibodies were detected using goat α-mouse antibodies conjugated to AlexaFluor® 568 (Invitrogen, Eugene, OR) at a dilution of 1:1,000 in PBS with 5% BSA. Finally, cells were washed 3x with PBS and once with water and mounted and mounted in Mowiol (Sigma-Aldrich, St. Louis, MO). Images were acquired on a Leica SP2 confocal microscope (Leica, Hicksville, NY).

Statistical analyses

Mann-Whitney tests were performed in order to compare two unpaired groups using the Graphpad Prism 5.0 software. *p value < 0.05, **p value < 0.01 and ***p value < 0.001 were considered to indicate a significant difference.

RESULTS

Identification of WEA-derived compounds with antiviral activity against HCV

The phytochemical investigation of the WEA extract resulted in the isolation and characterization of a new sesquiterpene lactone (compound 1, designated “grosheimol”), as well as five additional known compounds (compounds 2-6, Figure 1). The complete data that describe the structural elucidation, absolute configuration and quantitative determination of all compounds are available upon request. The anti-HCV activity of all six compounds was evaluated by using the highly permissive human hepatoma cell line Huh7/Scr and firefly luciferase reporter viruses based on the intra-genotypic genotype 2a chimera Jc1 (Luc-Jc1 (17), Fig. 2A). Briefly, the day of infection, Huh7/Scr cells were treated for 1h with 10 or 20 μM of each compound or dasatinib (at the same concentrations) (18), a known HCV entry inhibitor. The total WEA extract was included in this experiment (at 100 and 200 μg/ml) in order to compare its anti-HCV capacity with the isolated compounds. Then, compounds-containing media were removed and cells were infected with fresh media that contained Luc-Jc1 and compounds. At 4h post infection media were replaced again with fresh compounds-containing media and 72h after infection cells were assayed for firefly luciferase activity. Cell viability was monitored in parallel

with an ATP assay (19). As shown in Fig. 2B, all 6 molecules and the WEA extract inhibited HCV cell entry and/or RNA replication. Compounds 1 and 2 (grosheimol and cynaropicrin) exhibited the greater potency by inhibiting HCV more than 90% or 80%, respectively. For this, these compounds were characterized further with respect to their anti-HCV capacity.

Cynaropicrin and grosheimol potently inhibit HCV

To assess the potency of the isolated sesquiterpene lactones cynaropicrin and grosheimol against HCV, the Luc-Jc1 virus was used to estimate the half maximal effective concentration [EC_{50}], the half maximal cytotoxic concentration [CC_{50}] and the selectivity index [(CC_{50} / EC_{50}), SI] by performing a dose-response infection assay. Total WEA extract was included again in this assay in order to compare potency of isolated compounds vs. the crude extract. Dasatinib served again as positive control. The infection protocol and the compounds incubation period were similar to that described above. As shown in Fig. 3A-D, estimated EC_{50} for cynaropicrin was 1.3 μM [CC_{50}: 16.9 μM, and the selectivity index (SI):13.0], as compared with an EC_{50} of 1.0 μM for grosheimol [CC_{50}: 36.7 μM, SI: 36.7] and an EC_{50} of 1.5 μM for dasatinib [CC_{50}: 20.3 μM, SI: 13.5] in this assay. As expected, estimated values for total extract [EC_{50}: 299 μg/ml, CC_{50}: 686 μg/ml, SI: 2.3] indicate a less potent formulation in comparison to the isolated compounds.

Cynaropicrin and grosheimol inhibit HCVpp cell entry.

We then sought to determine in which step of the viral life cycle cynaropicrin and grosheimol exert their action; whether it was entry, replication/translation or assembly/release. Different HCV-based systems exist that allowed us to dissect the different steps of the viral life cycle (20).

To assess if cynaropicrin and grosheimol inhibit the entry step, we used the HCV pseudoparticle system (HCVpp). HCVpp is a well-established system for the study of HCV entry and neutralization (21, 22). They consist of lenti- or retroviral cores surrounded by an envelope displaying HCV E1E2 envelope glycoproteins. For consistency with the previous experiments, we used HCVpp that carry glycoproteins (genotype 2a, isolate J6CH) identical to those of the Luc-Jc1 virus. The day of infections, Huh7/Scr cells were treated for 1h with high concentrations of isolated compounds cynaropicrin (at 5 μM), grosheimol (at 20 μM) or the control dasatinib (at 5 μM). Then, cells were infected with HCVpp-compound mix at the same concentration. Six hours post infection, HCVpp-containing media was replaced with fresh media-compound mix and 72 h after infection, cells were assayed for renilla luciferase activity. HCVpp were inhibited in the presence of cynaropicrin (~70%), or grosheimol (~40%) (Fig.4A, left). These results indicate that both compounds act at least at the entry level. The positive control dasatinib also reduced infectivity levels of the HCVpp (~60%) as expected. Consistent with the results in Fig.2, neither grosheimol nor cynaropictin showed cytotoxity for the concentrations used in this assay (Fig. 4A, right). HCVpp are lenti-viral particles that harbor on their surface the HCV glycoproteins. Early entry steps of HCV are recapitulated by these particles but late entry steps are dependent on HIV replication mechanisms. To discriminate between an effect of cynaropicrin and grosheimol on HCV envelope protein functions and the process leading to lentiviral integration and expression, we performed the exact same experiment using pseudoparticles that harbor on their surface the glycoprotein of the vesicular stomatitis virus (VSV-G). As shown in Fig. 4B, only cynaropicrin showed a ~30% inhibition in the entry or expression of these particles. According to this result, we cannot exclude a low inhibitory capacity of cynaropicrin on entry of VSV-G or expression of HIV lenti-viral particles. However,

the results presented in Fig. 4A in combination with the results in previous figures point out a strong inhibitory capacity of this compound against HCV.

Cynaropicrin and grosheimol inhibit early steps of HCVcc entry

HCV entry is a multistep process that involves viral proteins and several cellular receptors. Cynaropicrin- and grosheimol-mediated inhibition of HCVpp could be a result of either a block in virus-cell binding, virus uptake or viral delivery into the cytoplasm. To characterize at which step of HCV entry grosheimol and cynaropicrin act, we investigated the inhibitory capacity of the different compounds when administered at different intervals during the early phase of infection (Fig. 5). We used again a high-titer reporter virus preparation of Luc-Jc1 and incubated it in the presence or absence of inhibitors with Huh7/Scr cells for 1 h at 4°C. Under these conditions, virus attaches to the cells but does not efficiently enter, thus permitting a rather synchronous infection when the inoculum is removed and cells are shifted to 37°C. Therefore, subsequent to virus attachment, unbound virus was washed away, cells were transferred to 37°C, and inhibitors were added either directly or 30, 60, 90, 120 or 240 min later for an interval of 4 h as indicated in Fig. 5A. Heparin, which is known to inhibit HCV attachment (17), dasatinib which is known to inhibit late steps in the entry process (18) and chlorpromazine which is a fusion inhibitor by causing clathrin lattices to assemble on endosomal membranes and at the same time prevents the assembly of coated pits at the plasma membrane (23), were used as controls. Under these conditions, we found that heparin inhibited the infection only when it was present during virus binding, as expected (Fig. 5B). Interestingly, both grosheimol and cynaropicrin inhibited virus binding and when added to cells at the point of temperature shift from 4^{o}C to 37°C, clearly indicating that these compounds play an inhibitory role during the

early steps of HCV entry. Dasatinib and chrorpromazine exhibited a strong inhibition when added early during infection while they kept their inhibitory capacity up to 120 min post shift at 37°C, confirming their role in late HCV entry events.

Cynaropicrin and grosheimol act directly on HCV particles

According to the previous results, grosheimol and cynaropicrin inhibit HCV during the early events of its entry. However, these compounds could be acting directly on the viral particle or could be mediating an effect on the host cell, or both. In order to address this issue, we performed time-of-addition experiments in which either the cells or the virus were pre-incubated for 1h with compounds prior to virus inoculation, or a virus-compound mix was directly added to the cells or compounds were added after 4h of virus inoculation (Fig. 6 left). Both, grosheimol and cynaropicrin interfered with HCV infection when viruses were incubated with the compounds and then compounds were present during virus inoculation (Fig. 6 right). Pre-exposition of the cells to the compounds or addition 4 h after infection did not result in HCV inhibition. These data corroborate the previous results and point to a direct HCV particle inhibition mechanism.

Cynaropicrin and grosheimol neither inhibit HCV RNA replication and/or translation nor HCV particle production

To investigate the impact of cynaropicrin and grosheimol in HCV RNA translation and/or replication, we transfected Huh7/Scr cells with a subgenomic JFH1 luciferase replicon (SGR-JFH1, Fig. 7A, top) (12). Subgenomic replicons are autonomous-replicating molecules that lack

structural proteins. A reporter gene (firefly luciferase) is expressed via the HCV IRES followed by the encephalomyocarditis IRES which drives the expression of non-structrural proteins (NS3-NS5B). As a result, the viral RNA is translated and replicates but does not encapsidate or produce new virions. Briefly, Huh7/Scr cells were electroporated with the SGR-JFH1 RNA. Cynaropicrin or grosheimol were added after 4h and luciferase activity was assayed after 48 hours. Changes in luciferase levels correlate with levels of HCV replication and/or translation. A 2'-modified nucleoside analog (2'-C-methyladenosine, 2'-C-Met) (24) was used as a positive control. As shown in Fig. 7A (bottom), none of the tested compounds inhibited HCV RNA replication and/or translation, while the control compound exerted a strong inhibitory effect in this assay, suggesting that the WEA isolated compounds do not act at these viral life cycle steps.

Cynaropicrin and grosheimol do not inhibit HCV particle production and egress

To investigate the potential role of the isolated sesquiterpene lactones in HCV particle production, we adapted and slightly modified a HCV particle production assay described by Menzel et. al. (25). Briefly, as shown in Fig. 7B top, Huh/Scr cells were electroporated with Luc-Jc1 virus and cultured for 40h. At this time point cells were extensively washed with PBS and fed with fresh medium containing cynaropicrin or grosheimol for 2h. The MAPK/ERK kinase inhibitor U0126, which has been shown to inhibit HCV particle production, served as the positive control. After this two-hour compound treatment, cells were washed again extensively and fed with fresh-medium for 6h to allow particle production post compound treatment. Finally, the treated medium was collected, clarified from cells by centrifugation and tested for particle production in naïve Huh7/Scr cells, while the effect of the compounds on HCV replication/translation and their cytotoxicity was measured in the electroporated cells by

luciferase assays. As shown in the bottom of Fig. 7B, neither cynaropicrin nor grosheimol inhibited HCV particle production in this assay in contrast to U0126, which showed inhibition without any effect on replication/translation. These data narrow down the effects of cynaropicrin and grosheimol at the entry level.

Cynaropicrin and grosheimol are active against all major HCV genotypes

HCV isolates have been classified into seven major genotypes (1-7), differing in their nucleotide sequence by around 30%, and a number of subtypes (a, b, and so on) with ~20% sequence divergence (26). HCV treatment efficacy is influenced by viral genotype, and even subtype in the case of HCV genotype 1 infection, and consequently treatment decisions are made taking this information into consideration (27). All previous experiments in this study were performed using viruses derived from the genotype 2a. In order to determine if WEA isolated compounds are also active against the other HCV genotypes, we used chimeric JFH-1 based reporter virus constructs, carrying renilla luciferase inserted at the NS5A gene and structural proteins from all major HCV genotypes and the most relevant genotype 1 subtypes: 1a (isolate TN), 1b (isolate J4), 2b (isolate J8), 3a (isolate S52), 4a (isolate ED43), 5a (isolate SA13), 6a (isolate HK6a) and 7a (isolate QC69) (Fig. 8A) (15). Similar dose-response inhibition experiments as described above were performed in order to evaluate the potency of cynaropicrin and grosheimol against multiple HCV genotypes. Importantly, infectivity was reduced in this treatment condition for all different genotypes (Fig. 8B), indicating that WEA isolated compounds inhibit HCV infection independently of viral genotype or subtype. Estimated EC_{50}, CC_{50} and SI for all major HCV genotypes are presented in Table 1.

Impact of cynaropicrin and grosheimol in HCV cell-cell transmission

HCV is transmitted between hepatocytes via classical cell entry using cell-free diffusion but also uses direct cell-cell transfer to infect neighboring cells (28). All previously described assays where cynaropicrin and grosheimol inhibited HCV entry were based on cell-free diffusion transmission. To investigate the capacity of these compounds to inhibit HCV transmission in a cell-cell assay, we developed a cell-cell HCV inhibition assay based on that published by Lupberger et. al. (18). In this assay (Fig. 9A), a co-culture of 2 different cell types takes place: Huh7/Scr cells infected with the Jc1 virus act as HCV donor cells while Huh7.5/EGFP-NLS-IPS cells act as acceptor cells (10). In the later cells, upon HCV infection, the NS3-4A protease cleaves the IPS-1 protein (IFN-β promoter stimulator protein 1), which normally localizes to mitochondria, and subsequently the enhanced fluorescent protein (EGFP) localizes to the nucleus due to its fusion to a nuclear localization signal (NLS). To avoid cell-free virus infection, the co-culture is overlaid with agarose. As shown in Fig. 9B and 9C, cynaropicrin and grosheimol efficiently inhibited cell-cell transmission while the solvent (DMSO) did not prevent HCV infection of the Huh7.5/EGFP-NLS-IPS cells. The reversible tyrosine kinase inhibitor erlotinib, which has been also shown to inhibit HCV cell-cell transmission (18), was used as positive control.

DISCUSSION

The current study demonstrates an anti-HCV activity of two isolated and characterized compounds from aqueous leaf extracts of the wild Egyptian artichoke (WEA). The first previously unknown compound was designated grosheimol while the second was identified to be cynaropicrin. Both compounds belong to the class of sesquiterpene lactones and exhibited pan-genotypic anti-HCV activity with EC_{50} values in the low micromolar range. They inhibit HCV entry into target cells, and thus represent interesting hit compounds for further drug development. These findings support the efforts to use folk medicinal plants as a source in the search for pharmacologically active compounds.

A number of promising natural products with anti-HCV activities have been described (for recent reviews see (29-32). They were of different origins, chemical structures and exerted their antiviral effects at different levels within the virus life cycle. Amongst these were several sesquiterpene lactones derived from parthenolide, an active component of feverfew that has been used against fever and migraine (33). An analysis of the structure-activity relationship of parthenolide and various analogs as inhibitors of HCV replication has been performed with a subgenomic HCV replicon system and has shown that the α-methylene-γ-lactone moiety was important for maximal antiviral activity (33). Albeit grosheimol and cynaropicrin inhibit HCV at the level of viral entry into target cells and not replication, they interestingly contain the same structural element as parthenolide. This suggests a functional meaning in the context of the viral entry process as well, and deserves further investigations.

HCV entry into hepatocytes is a complex, multi-step and well-orchestrated process that involves the HCV envelope glycoproteins E1 and E2 as well as several host factors (34). HCV entry is an attractive target for antiviral drug development because it offers the advantage of combating the infection at its initial steps before virus progeny is generated that can persist by ongoing replication. Presently, several entry inhibitors have been described and their mode-of-action has been determined in mouse models or in cell culture (35). Among them, a plant derived flavonoid (36) has been shown to inhibit the entry of all major HCV genotypes in vitro while the green tea polyphenol, epigallocatechin-3-gallate, has been shown to inhibit primarily HCV entry (37, 38) and secondarily HCV replication (39). Nonetheless, most entry inhibitors are in preclinical development and only 2 of them are presently in clinical phase I/IIa of development. These are the ITX5061 inhibitor of the scavenger receptor class B type I and erlotinib, a known compound that inhibits the epidermal growth factor alpha receptor. Very recently, the co-administration of the anti-SRBI mAb1671 with the DAA ciluprevir (protease inhibitor) in human-liver mice offered the first preclinical in vivo evidence that addition of an entry inhibitor to an anti-HCV DAA regimen restricts the breakthrough of DAA-resistant viruses (36). Thus, they may present in the future an appropriate solution for (i) preventing liver graft infections that are universally observed when seropositive patients are not treated prior to liver transplantation and for (ii) treating in combination with DAAs difficult-to-treat patients like those with liver cirrhosis and hepatocellular carcinoma.

Our data indicate that cynaropicrin and grosheimol prevent HCV life-cycle steps that include virus binding and early entry processes. Importantly, both compounds inhibited HCV only when viruses were pre-incubated with the compounds but not the cells. These findings strongly suggest

that both compounds act directly to viral particles and may prevent virus-receptor interactions. Considering the antiviral activity of grosheimol and cynaropicrin against all HCV genotypes, these compounds present very attractive pan-genotypic anti-HCV natural products. Nevertheless, further studies regarding their toxicity are required. In our studies, although the selectivity of both compounds was comparable to that obtained with the anti-cancer drug dasatinib which is a Bcr-Abl tyrosine kinase inhibitor, full inhibition of HCV infectivity was achieved at cytotoxic concentrations. Additional studies with derivatives of cynaropicrin and grosheimol might help to overcome these difficulties in the future.

Medicinal food plants including artichokes are in widespread use sometimes for centuries (40-44). Their medical benefit is often anecdotic and does not always hold up to strict scientific criteria (45). Nonetheless, numerous interesting compounds with a broad range of bioactivities have been isolated from them and they present interesting lead compounds for further development into specific pharmaceuticals. A good example is cynaropicrin which, besides its here described anti-HCV activity for the first time, is active against the protozoan parasite *Trypanosoma brucei* which causes human African trypanosomiasis (sleeping sickness) (46). It mediates the effect by targeting the trypanothione redox system of the parasite (47). Additionally, it has been shown that cynaropicrin exhibits inhibitory effects on the production of tumor necrosis factor-α which is a proinflammatory cytokine (48). Furthermore, its antihyperlipidemic (49), anticancer (50) and anti-photoaging (51) activitites indicate that cynaropicrin has a diverse pharmaceutic potential. Because (i) semisynthetic derivatives of cynaropicrin with improved bioactivity properties can be generated with relative ease (52) and (ii) the genes encoding its biosynthesis in plants start to be characterized (53) and thus may

become available for engineering of microorganisms for cynaropicrin biosynthesis, there seem to be a number of efficient production options to generate affordable pharmaceutical products.

In conclusion, cynaropicrin and grosheimol are two natural products within the aqueous WEA with potent anti-HCV activities. Their chemical properties and ease of isolation and modification make them interesting candidates for further development into early step HCV inhibitors.

ACKNOWLEDGEMENTS

The authors wish to thank Päivi Joensuu (Mass laboratory, Department of Chemistry, University of Oulu) and Dr. Ulrich Bergmann (Department of Biochemistry, University of Oulu) for technical assistance. The work was supported by FEDER and the Spanish Ministry of Economy and Competitiveness through grants BFU 2013-44629-R, SAF2013-46077-R and the "Maria de Maeztu" Programme for Units of Excellence in R&D (MDM-2014-0370).

FIGURE LEGENDS

Figure 1: Chemical structures of the natural compounds isolated from the WEA.

Figure 2: Screening of WEA-derived compounds for antiviral activity against HCV. (A) Schematic drawing of the Luc-Jc1 reporter virus genome used for the screening. UTR: untranslated region, Fluc: Firefly luciferase, EMCV: encephalomyocarditis virus, IRES: internal ribosomal entry site. (B) Huh7/Scr cells were seeded on 96 well plates, 1.2 x 10^4 cells/well, 16h prior to infections. The day of infections, cells were incubated with compounds for 1h in the indicated concentrations. Then, compounds-containing media were removed and cells were inoculated with Luc-Jc1 virus-compounds preparations at the same concentrations. Finally, virus-compounds preparations were replaced with fresh medium-compounds preparations and HCV infection efficiency was determined 72h post inoculation using firefly luciferase assays. Cell viability was measured in parallel using an ATP assay. All data were plotted as percentage relative to DMSO for both infectivity and cell viability. Data are expressed as mean values of four measurements of two biological replicates (±SEM).

Figure 3: Grosheimol and cynaropicrin inhibit HCV genotype 2a. Huh7/Scr cells were seeded on 96 well plates, 1.2 x 10^4 cells/well, 16h prior to infections. The day of infections cells were treated with increasing concentrations of (A) cynaropicrin, (B) grosheimol or (C) total artichoke-extract for 1 h. Then, compounds-containing media were removed and cells were infected with Luc-Jc1 virus-compounds mix at a multiplicity of infection (MOI) of 0.01

$TCID_{50}$/cell. The tyrosine kinase inhibitor dasatinib, a known HCV entry inhibitor, served as positive control (D). Virus-compounds mix were replaced 4 h post infection with fresh media-compounds mix and 72 h after infection cells were assayed for Firefly luciferase activity and the mean relative light units (RLU) were plotted as percentage relative to DMSO for both infectivity and cell viability. Half maximal Effective Concentration (EC_{50}) and half maximal Cytotoxic Concentration (CC_{50}) were estimated by non-linear regression of log inhibitor vs. normalized response and used to calculate the Selectivity Index (SI) value. Data are expressed as mean values of four measurements of two biological replicates (±SEM).

Figure 4: Cynaropicrin and grosheimol inhibit HCVpp cell entry. Huh7/Scr cells were seeded on 96 well plates, 1.2 x 10^4 cells/well, 16h prior to infections. The day of infections, cells were treated with 5 μM of cynaropicrin, 20 μM grosheimol or 5 μM dasatinib for 1 h. Then, compounds-containing media were removed and cells were infected with HCV pseudoparticles (HCVpp)-compound mix at the same concentration (A) or pseudotypes carrying on their surface the VSV-G (B). HCVpp were carrying identical glycoproteins (genotype 2a, isolate J6CF) to the Luc-Jc1 viruses. Pseudotypes-compounds mix were replaced 6 h post infection with fresh media-compounds mix and 72 h after infection cells were assayed for renilla luciferase activity and the mean relative light units (RLU) were plotted as percentage relative to DMSO for both infectivity and cell viability. Data are expressed as mean values of four measurements of two biological replicates (±SEM).

Figure 5: Kinetics of inhibitory activity exerted by cynaropicrin and grosheimol. (A) Schematic drawing of the experimental setup of the experiment depicted in panel B. Inhibition of Luc-Jc1 entry into Huh7/Scr cells by heparin (100 μg/ml), cynaropicrin (5 μM), grosheimol (20 μM), dasatinib (5 μM) and chlorpromazine (5 μg/ml) was compared using seven different experimental protocols (indicated by roman numerals i. through vii.). Virus binding to target cells was performed for 1 h at 4°C in the absence (ii. to vii.) or in the presence (i.) of compounds. Subsequently, cells were washed with PBS and shifted to 37°C to allow entry to proceed. Depending on the protocol, inhibitors were added directly or 30, 60, 90, 120 or 240 min thereafter (ii., iii., iv., v., vi., and vii, respectively). Dotted lines indicate the time interval during which an inhibitor was present; black arrows indicate the addition and removal of virus inoculum (B). Efficiency of infections using the protocols described in panel A was determined by luciferase assays 72 h post infection. The mean relative light units (RLU) were plotted as percentages relative to DMSO. Data are expressed as mean values of four measurements of two biological replicates (±SEM).

Figure 6: Mode-of-action analysis of cynaropicrin and grosheimol by time-of-addition experiments. Huh7/Scr cells were seeded into 96 well plates, 1.2 x 10^4 cells/well, 16 h prior to infections. The day of infections, cells were inoculated with Luc-Jc1 reporter viruses prepared in the absence of drugs. Cynaropicrin (5 μM) or grosheimol (20 μM) were added to the cells only before inoculation (black), were added to viruses and pre-incubated with them at 37^{o}C prior to inoculation (stripes), or selectively directly after inoculation (white) as schematically depicted at the left. Infectivity was determined 72h later by firefly luciferase assays and the mean relative light units (RLU) were plotted as percentage relative to DMSO for both infectivity and cell

viability. Data are expressed as mean values of four measurements of two biological replicates (±SEM).

Figure 7: Neither grosheimol nor cynaropicrin inhibit HCV translation/replication or particle production and egress. (A) Huh7/Scr cells were transfected by electroporation with the subgenomic firefly luciferase replicon depicted at the top. Electroporated cells were seeded on 96 well plates at a concentration of 1.2 x 10^4 cells/well. Compounds were added 4 h post transfection at a final concentration of 5 μM or 20 μM (for cynaropicrin and 2´-C-Met or grosheimol, respectively) and the levels of HCV RNA translation/replication were quantified by firefly luciferase assays 72 h post transfection. The 2'-modified nucleoside analog (2'-C-methyladenosine, 2'-C-Met) (24) was used as a positive control. Data are expressed as mean values of four measurements of two biological replicates (±SEM). (B) Schematic representation of the experimental procedure is depicted at the top. Huh-7/Scr cells were electroporated with Luc-Jc1 RNA and seeded into replicate tissue culture plates. Artichoke-derived compounds and the known HCV particle production inhibitor U0126, were added into the medium at 40 h post electroporation at the given concentrations. Two hours later, cells were washed 3 times with PBS to remove the compounds and fed for 6 h with fresh medium. Finally, at 48 h post electroporation supernatant was harvested and cells were lysed. HCV RNA replication in cells was measured by using a firefly luciferase reporter assay (top panel). The release of infectious particles was determined by inoculation of naïve cells with the collected culture fluids and determination of firefly luciferase activity in naïve cells 72 h after inoculation (bottom panel). Data are expressed as mean values of four measurements of two biological replicates (±SEM).

Figure 8: Grosheimol and cynaropicrin inhibit HCV of different genotypes. (A) A schematic drawing of the Renilla luciferase-carrying reporter virus genomes used for this screening. RLuc: renilla luciferase (B) Huh7/Scr cells were seeded on 96 well plates, 1.2 x 10^4 cells/well, 16 h prior to infections. The day of infections, cells were pre-incubated for 1h with compounds at the given concentrations. Then, compounds-containing media were removed and cells were inoculated with genotypes 1-7 virus-compounds preparations at the same concentrations. Finally, viruses-compounds preparations were replaced with fresh medium 4h post inoculation (containing compounds) and HCV infection efficiency was determined 72h post inoculation using renilla luciferase assays and is expressed as the percentage of DMSO control. Data are expressed as mean values of four measurements of two biological replicates (±SEM).

Figure 9: Grosheimol and cynaropicrin inhibit HCV cell-cell transmission. (A) Cell culture model system for analysis of HCV cell-cell transmission. In this assay, Huh7/Scr cells are infected with the Jc1 virus and function as HCV "donor" cells containing replicating HCVcc (stained by an anti-NS5A antibody and indicated in red) are co-cultured with HCV^- Huh7.5/EGFP-NLS-IPS "acceptor" cells for 24 h. In the later cells, upon HCV infection, the NS3-4A protease cleaves the IPS-1 protein which normally localizes to mitochondria, and subsequently the EGFP (indicated in green) localizes to the nucleus due to its fusion to a nuclear localization signal (NLS). Fluorescence microscopy is used to quantitate infected HCV^+ Huh7.5/EGFP-NLS-IPS "acceptor" cells (indicated by red and green), which are infected via cell-cell transmission and stained with anti-NS5A antibody (B) Huh7/Scr cells were infected with Jc1 virus at an MOI ~10 $TCID_{50}$/cell and 20h later seeded on 24 well plates, 5 x 10^4 cells/well. Simultaneously, Huh7.5/EGFP-NLS-IPS cells were seeded on the same 24 well plates

(co-culture) and cynaropicrin (10 μM), grosheimol (20 μM) or erlotinib (10 μM) were added to the wells. The addition of the compounds solvent (DMSO) served as negative control. Four hours later, medium was removed and cells were ovelaied with 1% agarose with fresh compounds. 24h later, the HCV infection was analysed by immunofluoresce of NS5A staining. Quantification of Huh7.5/EGFP-NLS-IPS cells was done in 3 independent wells by taking 3 independent pictures of different field of each well that contained at least 200 cells in total. Data are expressed as percentage to DMSO treated cells and represent mean values of the 3 independent fields of three biological replicates (±SEM). (C) Immunofluoresence analysis of NS5A protein (red) in co-culture of Huh7/Scr Jc1 infected cells with Huh7/EGFP-NLS-IPS, 24 h post co-culture and treated with erlotinib (10 μM), cynaropicrin (10 μM), grosheimol (20 μM) or mock treated (DMSO). Magnification 63x.

Table 1: Antiviral activity of grosheimol and cynaropicrin across all major HCV genotypes

	Cynaropicrin			Grosheimol		
HCV genotype	EC_{50} [μM]	CC_{50} [μM]	SI	EC_{50} [μM]	CC_{50} [μM]	SI
1a (TN)	0.4	16.8	40.5	2.7	76.4	28.3
1b (J4)	1.1	15.7	14.3	4.5	89.0	19.8
2b (J8)	0.7	19.4	27.7	7.2	103.3	14.3
3a (S52)	0.7	18.1	25.6	7.3	87.6	12.0
4a (ED43)	0.7	13.8	19.7	8.7	85.9	9.9
5a (SA13)	0.8	14.4	18.0	14.0	90.8	6.5
6a (HK6a)	0.7	17.8	25.4	6.3	92.2	14.6
7a (QC69)	1.4	15.1	10.8	5.7	86.7	15.2

REFERENCES

1. **Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, Simmonds P.** 2014. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology **59:**318-327.
2. **WHO** 2014, posting date. Fact sheet num. 164. [Online.]
3. **Lavanchy D.** 2009. The global burden of hepatitis C. Liver Int **29 Suppl 1:**74-81.
4. **Shepard CW, Finelli L, Alter MJ.** 2005. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis **5:**558-567.
5. **Webster DP, Klenerman P, Dusheiko GM.** 2015. Hepatitis C. The Lancet **doi:10.1016/S0140-6736(14)62401-6**.
6. **Franco S, Bellido R, Aparicio E, Canete N, Garcia-Retortillo M, Sola R, Tural C, Clotet B, Paredes R, Martinez MA.** 2011. Natural prevalence of HCV minority variants that are highly resistant to NS3/4A protease inhibitors. Journal of viral hepatitis **18:**e578-582.
7. **Franco S, Tural C, Nevot M, Molto J, Rockstroh JK, Clotet B, Martinez MA.** 2014. Detection of a sexually transmitted hepatitis C virus protease inhibitor-resistance variant in a human immunodeficiency virus-infected homosexual man. Gastroenterology **147:**599-601 e591.
8. **Muir AJ, Naggie S.** 2015. HCV treatment: is it possible to cure all HCV patients? Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association.
9. **Lattanzio V, Kroon PA, Linsalata V, Cardinali A.** 2009. Globe artichoke: A functional food and source of nutraceutical ingredients. Journal of Functional Foods **1:**131-144.

10. **Jones CT, Catanese MT, Law LMJ, Khetani SR, Syder AJ, Ploss A, Oh TS, Schoggins JW, MacDonald MR, Bhatia SN, Rice CM.** 2010. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. Nat Biotech **28:**167-171.

11. **Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R.** 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proceedings of the National Academy of Sciences of the United States of America **103:**7408-7413.

12. **Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, Wakita T.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology **125:**1808-1817.

13. **Neumann T, Hagmann I, Lohrengel S, Heil ML, Derdeyn CA, Krausslich HG, Dittmar MT.** 2005. T20-insensitive HIV-1 from naive patients exhibits high viral fitness in a novel dual-color competition assay on primary cells. Virology **333:**251-262.

14. **Koutsoudakis G, Dragun J, Perez-Del-Pulgar S, Coto-Llerena M, Mensa L, Crespo G, Gonzalez P, Navasa M, Forns X.** 2012. Interplay between basic residues of hepatitis C virus glycoprotein E2 with viral receptors, neutralizing antibodies and lipoproteins. PloS one **7:**e52651.

15. **Gottwein JM, Jensen TB, Mathiesen CK, Meuleman P, Serre SB, Lademann JB, Ghanem L, Scheel TK, Leroux-Roels G, Bukh J.** 2011. Development and application of hepatitis C reporter viruses with genotype 1 to 7 core-nonstructural protein 2 (NS2)

expressing fluorescent proteins or luciferase in modified JFH1 NS5A. J Virol **85:**8913-8928.

16. **van den Hoff MJ, Christoffels VM, Labruyere WT, Moorman AF, Lamers WH.** 1995. Electrotransfection with "intracellular" buffer. Methods in molecular biology **48:**185-197.

17. **Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T, Bartenschlager R.** 2006. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J Virol **80:**5308-5320.

18. **Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J, Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Doffoel M, Raffelsberger W, Poch O, McKeating JA, Brino L, Baumert TF.** 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nature medicine **17:**589-595.

19. **Niles AL, Moravec RA, Eric Hesselberth P, Scurria MA, Daily WJ, Riss TL.** 2007. A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. Analytical biochemistry **366:**197-206.

20. **Tellinghuisen TL, Evans MJ, von Hahn T, You S, Rice CM.** 2007. Studying hepatitis C virus: making the best of a bad virus. J Virol **81:**8853-8867.

21. **Bartosch B, Dubuisson J, Cosset FL.** 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med **197:**633-642.

22. **Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped

retroviral particles. Proceedings of the National Academy of Sciences of the United States of America **100:**7271-7276.

23. **Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C, Rouille Y.** 2006. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J Virol **80:**6964-6972.

24. **Carroll SS, Tomassini JE, Bosserman M, Getty K, Stahlhut MW, Eldrup AB, Bhat B, Hall D, Simcoe AL, LaFemina R, Rutkowski CA, Wolanski B, Yang Z, Migliaccio G, De Francesco R, Kuo LC, MacCoss M, Olsen DB.** 2003. Inhibition of hepatitis C virus RNA replication by 2'-modified nucleoside analogs. The Journal of biological chemistry **278:**11979-11984.

25. **Menzel N, Fischl W, Hueging K, Bankwitz D, Frentzen A, Haid S, Gentzsch J, Kaderali L, Bartenschlager R, Pietschmann T.** 2012. MAP-kinase regulated cytosolic phospholipase A2 activity is essential for production of infectious hepatitis C virus particles. PLoS pathogens **8:**e1002829.

26. **Nakano T, Lau GM, Lau GM, Sugiyama M, Mizokami M.** 2012. An updated analysis of hepatitis C virus genotypes and subtypes based on the complete coding region. Liver Int **32:**339-345.

27. **Lange CM, Zeuzem S.** 2013. Perspectives and challenges of interferon-free therapy for chronic hepatitis C. Journal of hepatology **58:**583-592.

28. **Timpe JM, Stamataki Z, Jennings A, Hu K, Farquhar MJ, Harris HJ, Schwarz A, Desombere I, Roels GL, Balfe P, McKeating JA.** 2008. Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. Hepatology **47:**17-24.

29. **Ashfaq UA, Idrees S.** 2014. Medicinal plants against hepatitis C virus. World journal of gastroenterology : WJG **20:**2941-2947.

30. **Calland N, Dubuisson J, Rouille Y, Seron K.** 2012. Hepatitis C virus and natural compounds: a new antiviral approach? Viruses **4:**2197-2217.

31. **Ishida Y, Takeshita M, Kataoka H.** 2014. Functional foods effective for hepatitis C: Identification of oligomeric proanthocyanidin and its action mechanism. World journal of hepatology **6:**870-879.

32. **Martinez JP, Sasse F, Bronstrup M, Diez J, Meyerhans A.** 2015. Antiviral drug discovery: broad-spectrum drugs from nature. Natural product reports **32:**29-48.

33. **Hwang DR, Wu YS, Chang CW, Lien TW, Chen WC, Tan UK, Hsu JTA, Hsieh HP.** 2006. Synthesis and anti-viral activity of a series of sesquiterpene lactones and analogues in the subgenomic HCV replicon system. Bioorganic and Medicinal Chemistry **14:**83-91.

34. **Ploss A, Evans MJ.** 2012. Hepatitis C virus host cell entry. Current opinion in virology **2:**14-19.

35. **Fofana I, Jilg N, Chung RT, Baumert TF.** 2014. Entry inhibitors and future treatment of hepatitis C. Antiviral research **104:**136-142.

36. **Haid S, Novodomska A, Gentzsch J, Grethe C, Geuenich S, Bankwitz D, Chhatwal P, Jannack B, Hennebelle T, Bailleul F, Keppler OT, Poenisch M, Bartenschlager R, Hernandez C, Lemasson M, Rosenberg AR, Wong-Staal F, Davioud-Charvet E, Pietschmann T.** 2012. A plant-derived flavonoid inhibits entry of all HCV genotypes into human hepatocytes. Gastroenterology **143:**213-222 e215.

37. **Ciesek S, von Hahn T, Colpitts CC, Schang LM, Friesland M, Steinmann J, Manns MP, Ott M, Wedemeyer H, Meuleman P, Pietschmann T, Steinmann E.** 2011. The

green tea polyphenol, epigallocatechin-3-gallate, inhibits hepatitis C virus entry. Hepatology **54:**1947-1955.

38. **Calland N, Albecka A, Belouzard S, Wychowski C, Duverlie G, Descamps V, Hober D, Dubuisson J, Rouillé Y, Séron K.** 2012. (−)-Epigallocatechin-3-gallate is a new inhibitor of hepatitis C virus entry. Hepatology **55:**720-729.

39. **Chen C, Qiu H, Gong J, Liu Q, Xiao H, Chen XW, Sun BL, Yang RG.** 2012. (-)-Epigallocatechin-3-gallate inhibits the replication cycle of hepatitis C virus. Archives of virology **157:**1301-1312.

40. **Guarrera PM, Savo V.** 2013. Perceived health properties of wild and cultivated food plants in local and popular traditions of Italy: A review. Journal of ethnopharmacology **146:**659-680.

41. **Mohanraj R, Sivasankar S.** 2014. Sweet potato (Ipomoea batatas [L.] Lam)--a valuable medicinal food: a review. Journal of medicinal food **17:**733-741.

42. **Ramalingum N, Mahomoodally MF.** 2014. The therapeutic potential of medicinal foods. Advances in pharmacological sciences **2014:**354264.

43. **Rondanelli M, Monteferrario F, Perna S, Faliva MA, Opizzi A.** 2013. Health-promoting properties of artichoke in preventing cardiovascular disease by its lipidic and glycemic-reducing action. Monaldi archives for chest disease = Archivio Monaldi per le malattie del torace / Fondazione clinica del lavoro, IRCCS [and] Istituto di clinica tisiologica e malattie apparato respiratorio, Universita di Napoli, Secondo ateneo **80:**17-26.

44. **Rubio L, Motilva MJ, Romero MP.** 2013. Recent advances in biologically active compounds in herbs and spices: a review of the most effective antioxidant and anti-inflammatory active principles. Critical reviews in food science and nutrition **53:**943-953.

45. **Wider B, Pittler MH, Thompson-Coon J, Ernst E.** 2013. Artichoke leaf extract for treating hypercholesterolaemia. The Cochrane database of systematic reviews **3:**CD003335.

46. **Zimmermann S, Kaiser M, Brun R, Hamburger M, Adams M.** 2012. Cynaropicrin: The first plant natural product with in vivo activity against trypanosoma brucei. Planta Medica **78:**553-556.

47. **Zimmermann S, Oufir M, Leroux A, Krauth-Siegel RL, Becker K, Kaiser M, Brun R, Hamburger M, Adams M.** 2013. Cynaropicrin targets the trypanothione redox system in Trypanosoma brucei. Bioorganic and Medicinal Chemistry **21:**7202-7209.

48. **Cho JY, Park J, Yoo ES, Baik KU, Jung JH, Lee J, Park MH.** 1998. Inhibitory effect of sesquiterpene lactones from Saussurea lappa on tumor necrosis factor-alpha production in murine macrophage-like cells. Planta Med **64:**594-597.

49. **Shimoda H, Ninomiya K, Nishida N, Yoshino T, Morikawa T, Matsuda H, Yoshikawa M.** 2003. Anti-hyperlipidemic sesquiterpenes and new sesquiterpene glycosides from the leaves of artichoke (Cynara scolymus L.): structure requirement and mode of action. Bioorganic & medicinal chemistry letters **13:**223-228.

50. **Cho JY, Kim AR, Jung JH, Chun T, Rhee MH, Yoo ES.** 2004. Cytotoxic and pro-apoptotic activities of cynaropicrin, a sesquiterpene lactone, on the viability of leukocyte cancer cell lines. European journal of pharmacology **492:**85-94.

51. **Tanaka YT, Tanaka K, Kojima H, Hamada T, Masutani T, Tsuboi M, Akao Y.** 2013. Cynaropicrin from Cynara scolymus L. suppresses photoaging of skin by inhibiting the transcription activity of nuclear factor-kappa B. Bioorganic and Medicinal Chemistry Letters **23:**518-523.

52. **Zimmermann S, Fouche G, De Mieri M, Yoshimoto Y, Usuki T, Nthambeleni R, Parkinson CJ, van der Westhuyzen C, Kaiser M, Hamburger M, Adams M.** 2014. Structure-activity relationship study of sesquiterpene lactones and their semi-synthetic amino derivatives as potential antitrypanosomal products. Molecules **19:**3523-3538.

53. **Menin B, Comino C, Portis E, Moglia A, Cankar K, Bouwmeester HJ, Lanteri S, Beekwilder J.** 2012. Genetic mapping and characterization of the globe artichoke (+)-germacrene A synthase gene, encoding the first dedicated enzyme for biosynthesis of the bitter sesquiterpene lactone cynaropicrin. Plant science : an international journal of experimental plant biology **190:**1-8.