Arch Virol (2016) 161:1169–1181
DOI 10.1007/s00705-016-2771-5

ORIGINAL ARTICLE

Khaya grandifoliola C.DC: a potential source of active ingredients against hepatitis C virus *in vitro*

Borris Rosnay Tietcheu Galani[1,2] · Marie-Emmanuelle Sahuc[3] · Gabriele Sass[4] · Frédéric Nico Njayou[1] · Christine Loscher[4] · Pierre Mkounga[5] · Gaspard Deloison[3] · Priscille Brodin[3] · Yves Rouillé[3] · Gisa Tiegs[4] · Karin Séron[3] · Paul Fewou Moundipa[1]

Received: 23 August 2015 / Accepted: 24 January 2016 / Published online: 3 February 2016

Abstract In this study, we examined the antiviral properties of *Khaya grandifoliola* C.DC (Meliaceae) on the hepatitis C virus (HCV) life cycle *in vitro* and identified some of the chemical constituents contained in the fraction with the most antiviral activity. Dried bark powder was extracted by maceration in a methylene chloride/methanol (MCM) system (50:50; v/v) and separated on silica gel by flash chromatography. Infection and replication rates in Huh-7 cells were investigated by luciferase reporter assay and indirect immunofluorescence assay using subgenomic replicons, HCV pseudotyped particles, and cell-culture-derived HCV (HCVcc), respectively. Cell viability was assessed by MTT assay, and cellular gene expression was analysed by qRT-PCR. The chemical composition of the fraction with the most antiviral activity was analysed by coupled gas chromatography and mass spectrometry (GC-MS). Five fractions of different polarities (F0-F100) were obtained from the MCM extract. One fraction (KgF25) showed the strongest antiviral effect on LucUbiNeoET replicons at nontoxic concentrations. Tested at 100 μg/mL, KgF25 had a high inhibitory effect on HCV replication, comparable to that of 0.01 μM daclatasvir or 1 μM telaprevir. This fraction also inhibited HCVcc infection by mostly targeting the entry step. KgF25 inhibited HCV entry in a pan-genotypic manner by directly inactivating free viral particles. Its antiviral effects were mediated by the transcriptional upregulation of the haem oxygenase-1 gene and interferon antiviral response. Three constituents, namely, benzene, 1,1′-(oxydiethylidene)bis (1), carbamic acid, (4-methylphenyl)-, 1-phenyl (2), and 6-phenyl, 4-(1′-oxyethylphenyl) hexene (3), were identified from the active fraction KgF25 by GC-MS. *Khaya grandifoliola* contains ingredients capable of acting on different steps of the HCV life cycle.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-016-2771-5) contains supplementary material, which is available to authorized users.

✉ Paul Fewou Moundipa
pmoundipa@hotmail.com

[1] Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, University of Yaounde I, PO Box 812, Yaounde, Cameroon

[2] Laboratory of Applied Biochemistry, Department of Biological Sciences, Faculty of Science, University of Ngaoundéré, Ngaoundéré, Cameroon

[3] CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019, UMR 8204, CIIL, Centre d'Infection et d'Immunité de Lille, Univ. Lille, 59000 Lille, France

[4] Institute of Experimental Immunology and Hepatology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

[5] Department of Organic Chemistry, Faculty of science, University of Yaounde I, Yaounde, Cameroon

Abbreviations

BCP	Boceprevir
CC_{50}	Cytotoxic concentration 50
CCLR	Cell culture lysis reagent
EGCG	Epigallocatechin-3-gallate
GC/MS	Gas chromatography/mass spectrometry
HCV	Hepatitis C virus
HCVcc	Cell-culture-derived hepatitis C virions
HCVpp	Pseudotyped particles of hepatitis C virus
IC_{50}	50 % inhibitory concentration
JFH-1	Japanese fulminant hepatitis 1
Kg	*Khaya grandifoliola*
KgCE	*Khaya grandifoliola* crude extract
KgF25	25 % methanol fraction of *Khaya grandifoliola*

LAR	Luciferase assay reagent
MCM	Methylene chloride/methanol
MOI	Multiplicity of infection
Nrf-2	Nuclear factor erythoid 2-related factor-2
NS	Nonstructural
PEG-IFN	Pegylated interferon
qRT-PCR	Quantitative real-time-polymerase chain reaction
SVR	Sustained virological response

Introduction

Hepatitis C virus (HCV) infection is a leading cause of chronic liver diseases. More than 170 million people worldwide are chronically infected by HCV, and about 500,000 die each year from hepatitis-C-related diseases [42]. In Cameroon, according to some epidemiological studies, about 14 % of individuals are affected [28] and HCV-related hepatocellular carcinoma is more prevalent among people older than 60 years, particularly males [2]. For the past decade, treatment of hepatitis C has been based on a combination of pegylated interferon alpha (PEG-IFNα) and ribavirin. In 2011, the efficacy of this treatment in genotype 1 patients was improved by including telaprevir or boceprevir, two protease inhibitors that have been approved by the US Food and Drug Administration (FDA) [23]. Recent progress in HCV therapy has led to the approval of new antiviral medicines that can cure hepatitis C in 8-12 weeks with high sustained virological response (SVR) rates. Among these, sofosbuvir, simeprevir and ledipasvir are some of the most interesting molecules, with an SVR higher than 90 % [9]. These compounds belong to the direct antiviral agents group and specifically target nonstructural proteins of HCV, such as NS3, NS5A or NS5B. Currently, access to the newly approved drugs is low, especially in developing countries, where the combination of PEG-IFNα and ribavirin, with moderate anti-HCV activity, is still used [41]. Furthermore, resistance due to viral mutations and multiple side effects due to PEG-IFN make it necessary to explore other antiviral agents that might reinforce the therapeutic landscape. Medicinal plants are a potential natural source of antiviral compounds. Natural molecules, including tannic acid, epigallocatechin-3-gallate (EGCG), honokiol, ladanein, silymarin, lucidone, and 3-hydroxycarulignan have recently been reported to possess potent anti-HCV activity *in vitro*, inhibiting entry, replication or secretion steps [10, 13, 14, 19, 27, 31, 44, 52]. Previous studies with a commercial silymarin-derived product clinically demonstrated significant reduction of viral loads in HCV-infected patients [44]. *Khaya grandifoliola* C.DC (*K. grandifoliola*), also called African mahogany, is a plant belonging to the family Meliaceae. *K. grandifoliola is* widely distributed from western Africa to the Guinean coast. In Cameroon, the plant is called *Fa'-tutu* in the Bamun language (West Cameroon) or *Dalehi* in North Cameroon. *K. grandifoliola* is used in folk medicine for the treatment of malaria, gonorrhea, skin diseases, diarrhea and gastric ulcers. The trunk and roots are the most-used parts. Previous studies on this plant have found antimalarial [33], antibacterial [48], anti-ulcer [40], anti-anaemic [1], anti-inflammatory [17], antifungal [43], hypoglycemic, and hypocholesterolemic [7] activity. In addition, mollusicidal [34], desmutagenic and antimutagenic [21], antioxidant [36], cytotoxic and hepato-protective effects [20, 38] have also been reported. Toxicological studies have been performed on the aqueous bark extracts, and subchronic toxicity has been reported for a dose of 500 mg/kg body weight in rats [8]. Phytochemical studies have led to the isolation and characterization of 11 limonoids, including grandifotane A [54], methylangolensate, gedunin, 7-deacetylkhivorine, 1-deacetylkhivorine and 6-acetylswietenolide, which showed anti-plasmodial effects [4, 33]. Other compounds were also identified, including catechin [43], quercetin-3-O-glucoside, quercetin 3-O rhamnoside and several methyl esters of fatty acids [20, 21]. However, to our knowledge, antiviral properties have not yet been demonstrated.

In this study, we report the antiviral activity of *K. grandifoliola* (Kg) fractions against HCV infection. The effect of Kg crude extract and its fractions, and more precisely, the KgF25 fraction, on HCV replication and cellular gene expression was demonstrated using a subgenomic replicon. The fraction KgF25 was also identified as a potent inhibitory agent against the HCV viral life cycle that specifically targets viral entry by inactivating free HCV virions of different genotypes. The bio-guided subfractionation and phytochemical characterisation of KgF25 by gas chromatography coupled to mass spectrometry led to the identification of new structurally defined compounds that might interfere with the HCV life cycle.

Materials and methods

Plant collection

Trunk barks of *K. grandifoliola* were harvested in May 2010 in the locality of Foumban (West Region of Cameroon). Botanical identification was done by Mr. Victor Nana of the National Herbarium of Cameroon (IRAD, Yaounde), and a voucher specimen was deposited under the reference number 23434 YA.

Reagents and material

Silica gel 230–400 mesh (Merck) and silica gel 70–230 mesh (Merck) were used for flash and column chromatography, while precoated aluminum-backed silica gel 60 F254 sheets ALUGRAM XTRA SIL were used for thin-layer chromatography (TLC). Spots were visualized under UV light (254 and 366 nm). Dulbecco's modified Eagle's medium (DMEM), GlutaMAX-I, and goat and fetal calf sera were purchased from Invitrogen (Carlsbad, CA, USA). 4,6-Diamidino-2-phenylindole (DAPI) was obtained from Molecular Probes (Life Technologies). EGCG was obtained from Calbiochem (Merck Chemicals, Darmstadt, Germany). Stocks were resuspended in dimethyl sulfoxide (DMSO) at 0.5 M for EGCG and 250 mg/mL for plant extracts. Boceprevir was kindly provided by Philippe Halfon (Hôpital Européen, Laboratoire Alphabio, Marseille, France). Telaprevir (VX-950) was purchased from Janssen-Cilag International (Beerse, Belgium) and Daclatasvir (BMS-790052) was purchased from Sellekchem (Houston, USA). These antivirals were dissolved in DMSO before use.

Cell lines and antibodies

Huh 7-derived hepatoma cells lines (LucUbiNeo-ET and Huh 5-15 cells) stably transfected with an HCV subgenomic replicon, Con1 (HCV genotype 1b; [32]), were used as replicon cell lines. In LucUbiNeo-ET, the luciferase gene of the firefly *Photinus pyralis* was included [26], allowing the detection of viral replication by luciferase reporter assay, while in Huh5-15 cells, this reporter gene was absent. Huh-7 cells were used for tests with HCVcc particles. Mouse anti-E1 monoclonal antibody (MAb)A4 [16] was produced *in vitro*. Cy3-conjugated goat anti-mouse IgG was obtained from Jackson Immunoresearch (West Grove, PA, USA).

Plant extraction and structure elucidation

Extraction and fractionation

The collected trunk barks were washed with water, air-dried and ground. One kg of the powder was extracted by maceration at room temperature with 3 L of a methylene chloride-methanol (MCM) solvent system (1:1, v/v) until exhaustion. The solvent was evaporated under reduced pressure using a rotary evaporator to yield 270 g of crude extract. A portion of the extract (268 g) was fractionated by flash chromatography over silica gel (70-230 mesh, Merck) by eluting with a gradient of increasing polarity in the MCM system 1:0 to 0:1, resulting in the collection of five fractions after TLC analysis. These fractions were labeled KgF0 (MCM 1:0, v/v), KgF5 (MCM 0.95:0.05, v/v), KgF10 (MCM 0.90:0.10, v/v), KgF25 (MCM 0.75:0.25, v/v), and KgF100 (MCM 0:1, v/v) according to the methanol gradient used in the solvent system. All of these fractions were tested *in vitro* against HCV genotype 1b subgenomic replicons.

Gas chromatography/mass spectrometry analyses

The composition of KgF25 was also analysed by gas chromatography/mass spectrometry (GC/MS). Samples (1 μL) were injected into a 150 RCN column with a split ratio of 1:1, using an Agilent auto-injector. The separation was done using helium as the carrier gas at a constant flow of 2 mL/min. The temperature was raised from 80°-220 °C during GC. The compounds were identified by electron ionisation–mass spectrometry (VG 70SE double focusing sector field mass spectrometer, VG Analytical Inc. Ltd., Manchester, UK). Mass spectra of all detected compounds were compared with those in the National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, USA) library of 2012. The plant extracts were dissolved in DMSO (Sigma-Aldrich) as stock solutions and further diluted in complete DMEM immediately before experiments to reach a final DMSO concentration of 0.5 % in replicon systems, or less than 0.01 % in HCVcc systems.

Cell culture

Replicon cell lines were cultured in Dulbecco's modified Eagle medium (Invitrogen GmbH, Karlsruhe, Germany) containing 10 % fetal bovine serum, 100 UI/ml of penicellin, 100 μg/mL of streptomycin and G418 (0.5 % for Huh-5-15, 0.25 % for LucUbiNeo-ET). Cells were seeded in tissue culture flasks with ventilated filter caps at 37 °C in a humidified atmosphere, 20 % O_2 and 5 % CO_2. Huh-7 was cultured in complete DMEM using Petri dishes of 10-cm diameter. Cells were incubated until confluence in humidified atmosphere of 5 % CO_2 and sub-cultivated every 3 days.

Luciferase-based replication assay

LucUbiNeo-ET cells were seeded in 24-well plates (1×10^5 cells/well) and incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in the presence or absence of various concentrations of antiviral agents (0.01-1 μM) or the Kg crude extract or fractions (0-1000 μg/mL). After 24 or 72 h of incubation, the medium was removed, cells were washed once with 500 μL of 1× PBS, and 120 μL of cell culture lysis reagent (CCLR; 1:5 dilution: 25 mM Tris-phosphate [pH 7.8], 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N0,N0-tetraacetic acid, 10 % glycerol, and 1 % Triton X-100) was added. The lysates were incubated for 10 min on ice and centrifuged at 14,000 rpm for 1 min at 4 °C). Luciferase activity was measured in the

supernatants using luciferase assay reagent (LAR). Bioluminescence was instantly detected and quantified using a luminometer (Tecan® Infinite M200). Luciferase values were normalized to the protein content of each sample, which was quantified by the Bradford method [6].

Analysis of cellular gene expression

Huh-5-15 cells were treated with the KgF25 fraction at 500 μg/mL for 24 h. RNA isolation and quantitative real-time RT-PCR (qRT-PCR) were performed as described previously [30]. Relative mRNA levels of virus and cellular genes investigated were obtained by normalization against the cellular endogenous ATP synthase gene. Primers used in the study are listed in Table 1.

HCVcc production

The virus JFH1-CSN6A4 used in this study was based on HCV "Japanese Fulminant hepatitis-1" (JFH1) of genotype 2a and contained a reconstituted A4 epitope in E1 and cell-culture-adaptive mutations [18]. Huh-7 cells were transfected with JFH1-CSN6-A4 to produce cell-derived infectious HCV particles (HCVcc), and the conditioned medium was collected to harvest viral particles. The protocol used has been described elsewhere [15, 18]. The virus titres of culture supernatants were determined by measuring infectivity by indirect immunofluorescence assay using anti-E1 monoclonal antibodies. The titer of each stock solution was 6×10^5 focus-forming units/mL. Supernatants were aliquoted and stored at -80 °C until use.

Virus assays

The effect of Kg crude extract and KgF25 on continuous HCVcc infection was examined. Plant extracts and EGCG were resuspended in complete DMEM with the viral inoculum to achieve a final concentration of 25 μg/mL and 50 μM, respectively. Huh-7 cells were seeded the night before in 96-well plates at 6000 cells and then inoculated for 2 h with 100 μL of the viral inoculum containing plant extracts at a multiplicity of infection (MOI) of 1. The medium was then replaced by 100 μL of fresh medium containing only the extracts. At 28 h postinfection, cells were fixed in ice-cold methanol and used for immunofluorescence analysis.

Effect of plant samples on virus entry and replication steps

Three sets of experiments were conducted in parallel in order to determine the mode of action of crude extract (KgCE) and KgF25 on HCVcc infection. (i) The effect on the entry step was determined by inoculating Huh-7 cells with HCV in the presence of plant sample for 2 h and replacing the medium with fresh medium without extracts for 28 h. (ii) To assess the effect at the post-entry step, HCV was applied to the cells in the absence of the sample for 2 h. The virus was removed, and cells were refed with fresh medium containing the plant sample for 28 h. (iii) EGCG, an inhibitor of the entry step [10], was added as a control at 50 μM during the 2-h inoculation step. Boceprevir, a viral NS3/4A protease inhibitor [46] that inhibits the replication step, was added as a control at 0.5 μM during the 28-h post-inoculation step.

Effect of KgF25 on HCV reporter pseudovirus

Pseudoparticles with either HCV E1E2 envelope proteins (HCVpp) of different genotypes (1b, 2a, 3a, 4 and 6) or the G envelope glycoprotein of vesicular stomatis virus (VSV-Gpp) were produced as described previously [3]. Huh-7 cells were seeded in 24-well plates at a density of 1×10^5 cells/well for overnight incubation and then simultaneously treated with an equal volume of medium containing different pseudoparticles and the KgF25 fraction (25 μg/mL) or DMSO (0.1 %). After 2 h of incubation, the inoculum was removed, and cells were further incubated with fresh

Table 1 Oligonucleotide sequence of primers used in qRT-PCR

Oligonucleotide name	Sequence 5′-3′
5′HO-1	5′-GAG ATT GAG CGC AAC AAG GAG-3′
3′HO-1	5′-CTG ACT GCG GGA GTC ATC TC-3′
5′OAS 3	5′-CAC TGA CAT CCC AGA CGA TG-3′
3′OAS 3	5′-GAT CAG GCT CTT CAG CTT GG-3′
5′OAS 1	5′-CAA GCT CAA GAG CCT CAT CC-3′
3′OAS 1	5′-TGG GCT GTG TTG AAA TGT GT-3′
5′HCV 214	5′-TGC GGA ACC GGT GAG TAC-3′
3′HCV 214	5′-AGG TTT AGG ATT CGT GCT CAT-3′
5′ATP synthase	5′-ATT GCC ATC TTG GGT ATG GA-3′
3′ATP synthase	5′-AAT GGG TCC CAC CAT GT-3′

DMEM for 46 h. Then, the cells were lysed in 100 µL of fivefold-diluted CCLR to measure the luciferase activity (Promega) using a Centro XS LB 960 microplate luminometer from Berthold Technologies (Bad Wildbad, Germany). The mean luciferase value of DMSO-treated cells was defined as 100 %, and the relative luciferase activity of KgF25-treated cells was expressed as a percentage of the DMSO group.

Pre-incubation experiments with virus

To determine whether the antiviral effect of Kg crude extract and KgF25 on HCV entry is due to the inactivation of viral particles, tenfold-concentrated HCVcc viral inoculum was pre-incubated with Kg crude extract and KgF25 for 1 h at a concentration of 25 µg/mL at 37 °C in a humidified atmosphere with 5 % CO_2. After this pre-incubation time, the inoculum was diluted tenfold to achieve a final concentration of 2.5 µg of plant extracts per mL. One hundred microliters of this suspension was applied to Huh-7 cells for 2 h. In parallel, Huh-7 cells were also treated with a viral inoculum that was not pre-incubated, containing Kg extracts at 25 and 2.5 µg/mL. At 28 h postinfection, cells were used for IF analysis to quantify the level of infection.

Immunofluorescence detection and quantification

After infection, cells were fixed in ice-cold methanol and then processed for immunofluorescent detection of E1 envelope glycoprotein with Cy3-labeled antibody as described previously [45]. Nuclei were stained with 1 µg of DAPI per mL. The infection of cultured cells by HCVcc resulted in focus formation that appeared as clusters of E1-positive cells. The foci were counted, and confocal images were recorded on an automated confocal microscope (IN Cell Analyzer 6000, GE Healthcare Life Sciences) using a 20X objective as described previously [11]. For quantification, four fields per well were recorded randomly. Each image was then processed using Columbus image analysis software (PerkinElmer). Nuclei were first segmented, and the cytoplasm region was extrapolated on the basis of DAPI staining. Cells labeled with anti-E1 antibody (in green) were counted as infected cells. The total number of cells was determined by counting DAPI-labeled nuclei (blue). Infection rates were scored as the ratio of the infected cells to total cells. The infection rate of DMSO-treated cells was taken as 100 %, and the relative number of infected cells was then calculated as a percentage of the number of infected DMSO-treated cells. The number of cells per well and MOI were calculated to obtain 30 to 40 % infected cells at 28 h postinfection, allowing automated quantification.

Cytotoxicity assay

The effect of Kg extracts on the viability of LucUbiNeo-ET replicon cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay kit (Promega, Mannheim, Germany) according to the manufacturer's instructions. The effect on Huh-7 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-ecarboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Briefly, Huh-7 cells were seeded in 96-well plates at a density of 6000 cells/well. After an overnight incubation, cells were incubated in 100 µL of culture medium containing plant extracts at different concentrations (three wells per condition) for either 24, 48, or 72 h in an incubator at 37 °C with 5 % CO_2. An MTS-based viability assay (CellTiter 96 Aqueous non-radioactive cell proliferation assay, Promega) was conducted as recommended by the manufacturer, and the amount of formazan was quantified by measuring absorbance at 490 nm using an ELISA plate reader (ELX 808 Bio-Tek Instruments Inc).

Statistical analysis

Results are presented as the mean ± SEM of triplicate experiments. The data were analyzed using one-way analysis of variance (ANOVA) followed by the Dunnett *post hoc* test. Grouped data were compared using two-way ANOVA followed by the Bonferroni test. Comparisons were made between each treated group and the untreated group (DMSO control). Differences between treated and control groups were considered significant for any p-value <0.05. P-values are indicated whenever significant differences were observed. These analyses were performed using Prism 5.0 statistical software (Graph Pad Inc.).

Results

Kg contains active constituents that suppress HCV replication in replicon systems

To examine the anti-HCV effect of Kg, we first used LucUbiNeo-ET replicon cells that contain genotype 1b subgenomic HCV RNA. The cells were incubated in the presence of plant crude extract (KgCE) at various concentrations from 100-1000 µg/mL for 24 or 72 h, and luciferase activity was then measured to determine the level of HCV RNA expression. Relative to the DMSO-treated group, KgCE caused a dose-dependent and significant reduction of HCV replication after 24 and 72 h of treatment (Fig. 1a). The screening of fractions derived from this extract led to the identification of KgF25 as the most active fraction, with 90 % of inhibition at 500 µg/mL

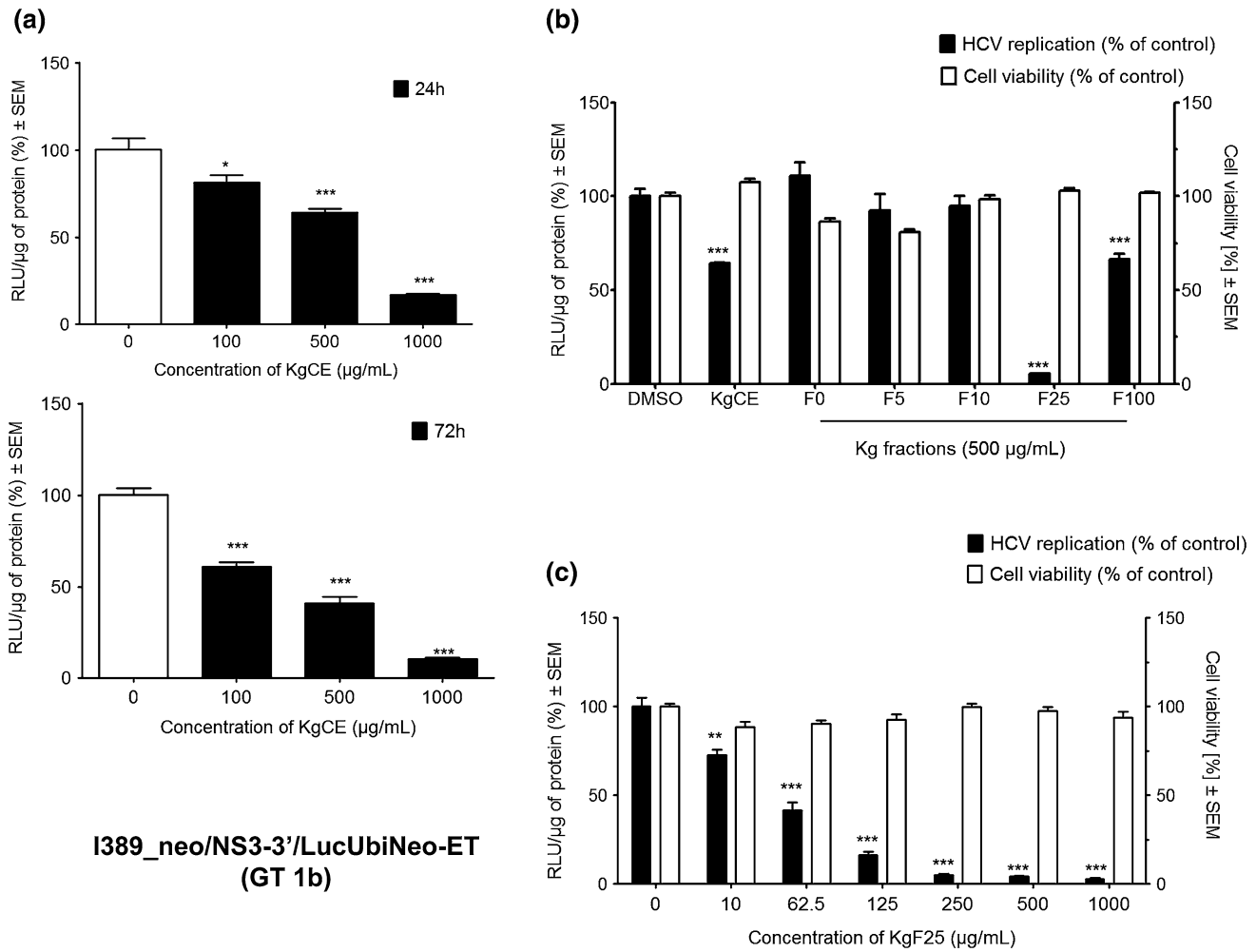

Fig. 1 *Khaya grandifoliola* bark extract inhibits HCV replication in LucUbiNeo-ET replicon cells. (a) Replicon cells were incubated in the presence of various concentrations of Kg crude extract (100-1000 µg/mL) for 24 h and 72 h. The effect on replication was assessed by measuring luciferase reporter activity expressed as relative luciferase (RLU/µg of protein) in comparison with the control group. **, $p < 0.01$; ***, $p < 0.001$ vs. the control group, using the Bonferroni test. (**b**) Replicon cells were treated with Kg fractions containing different proportions of the methylene chloride/methanol system (F0, fraction with 0 % methanol and 100 % methylene chloride; F5, the 5 % methanol fraction; F10, the 10 % methanol fraction; F25, the 25 % methanol fraction; F100, the 100 % methanol fraction) at 500 µg/mL for 24 h. Cell viability was determined by MTT assay and replication by luciferase reporter assay. (**c**) Dose-dependent inhibition of viral replication by KgF25 in replicon cells after 24 h of incubation

($p < 0.001$) (Fig. 1b). A dose-dependent inhibition of the HCV replication was found with this fraction (Fig. 1c), with an IC_{50} value of 27.17 µg/mL. Moreover, no significant decrease in cell viability was observed at the tested concentrations (Fig. 1c).

KgF25 strongly inhibits HCVcc infection, mostly by interfering with the entry step

To further characterize the anti-HCV activity of KgF25, the effect of this fraction was tested at 25 µg/mL on the HCVcc system by adding the plant extract at various times of the infectious process. The plant extract was added either during the inoculation step representing the entry step, during the post-inoculation (replication) step, or continuously during the enitre HCV life cycle. The level of infection was then quantified by indirect immunofluorescence assay using anti-E1 monoclonal antibodies and confocal microscopy using automated image analysis. Compared to DMSO-treated cells, KgF25 significantly inhibited HCV infection ($p < 0.001$) by 84 % when added continuously during the whole viral cycle. This effect was found similar to those of EGCG (50 µM) and boceprevir (0.5 µM) used as positive controls (Fig. 2a). Although the infection efficiency was significantly ($p < 0.01$) reduced by 29.11 % during the replication step (post-inoculation

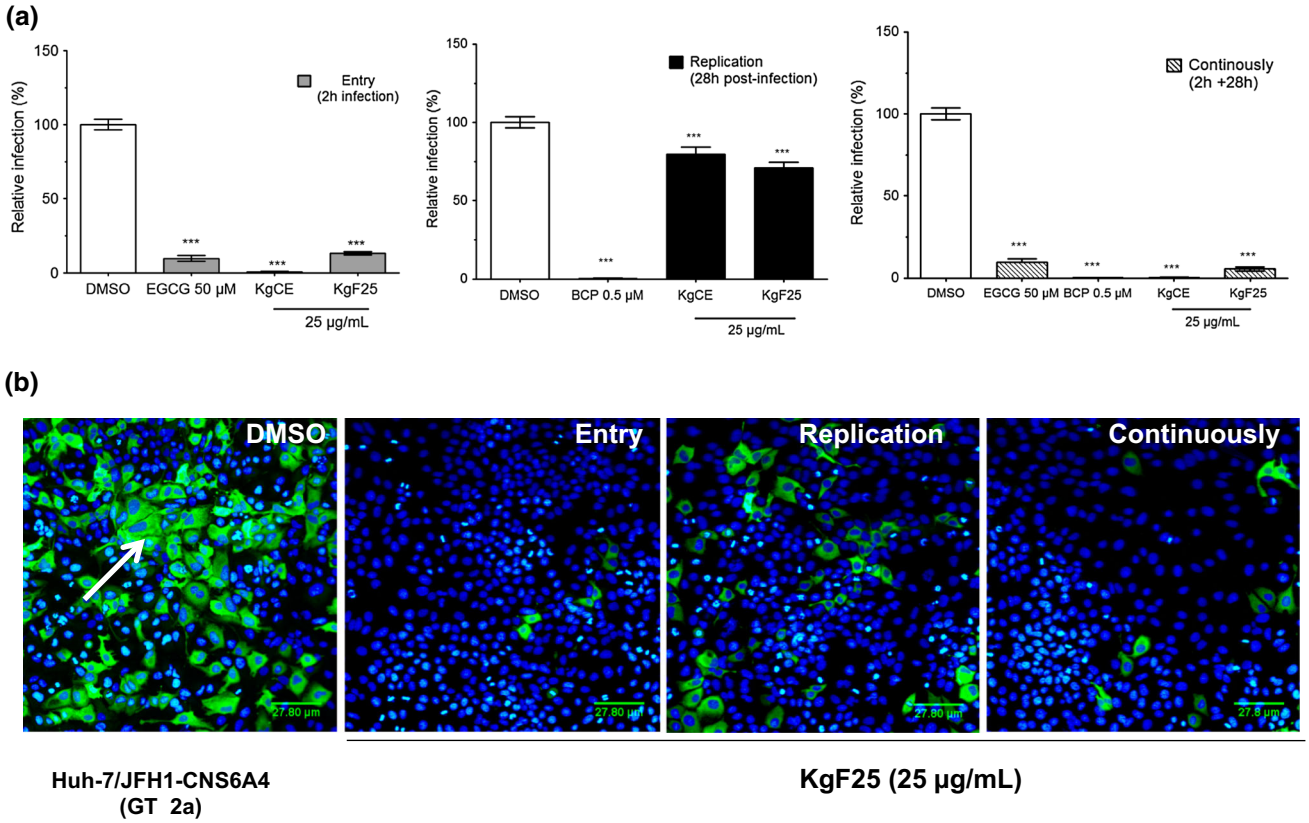

Fig. 2 The Kg crude extract and the KgF25 fraction strongly target viral entry to inhibit HCVcc infection in Huh-7 cells (**a**) Huh-7 cells were infected with HCVcc particles for 2 h in the presence of 25 µg of the KgF25 fraction per mL, 0.01 % DMSO (entry), 50 µM EGCG, or 0.5 µM boceprevir. After 2 h, the viral inoculum was removed, and cells were treated with plant extract or test compounds for 28 h post-inoculation (replication). Cells were also treated in continuously for 30 h with the KgF25 extract. The infection rates were quantified by immunofluorescence staining using anti-E1 monoclonal antibodies. **, $p < 0.01$; ***, $p < 0.001$ vs. the DMSO-treated group using the Dunnett test. (**b**) Confocal fluorescence micrographs were taken from four random fields per sample in triplicate in each experiment, and the cells expressing glycoprotein E1 (in green and indicated by a white arrow) were counted. Nuclei were stained with DAPI (in blue)

step) by KgF25 treatment, a greater decrease in infection (82.11 %; $p < 0.001$) was observed during the entry step (Fig. 2a). Confocal microscopic analysis clearly showed a time-dependent reduction in the number of infected cells, suggesting a decrease in the amount of E1 (Fig. 2b). This inhibition of E1 by KgF25 observed in the immunofluorescence assay was confirmed by TaqMan RT-PCR, which showed a significant decrease ($p < 0.01$) in genomic HCV RNA expression in HCVcc-infected cells (Online resource 1). At the concentration where KgF25 or KgCE was found to be active, no significant decrease in cell viability was observed (data not shown). Using MTT assay, we also analysed the dose-dependent cytotoxic effect of this fraction and KgCE. Both showed similar cytotoxic concentration (CC_{50}) values (Table 2). However, KgCE was found more active against HCV entry than KgF25. An IC_{50} value of 2.33 ± 0.33 µg/mL was obtained with KgCE, and 11.43 ± 1.80 µg/mL with KgF25. The selectivity index of KgCE was clearly higher than those of KgF25 and EGCG. which were used as positive controls (Table 2).

Table 2 Selectivity index (SI) of KgCE, KgF25 and EGCG on HCVcc entry in Huh-7 cells

	CC_{50} (µg/mL)	IC_{50} (µg/mL)	Index CC_{50}/IC_{50}
KgCE	163.4 ± 47.1	2.33 ± 0.33	70
KgF25	192.1 ± 29.7	11.43 ± 1.80	16
EGCG	(>91.67)	2.29	>40

Huh-7 cells were incubated with HCVcc in the presence of various concentrations of Kg crude extract (KgCE), the F25 fraction (KgF25) and epigallocatechin-3-gallate (EGCG) for 2 h. Then, medium was changed and cells were incubated for 28 h. An immunofluorescence assay was used to measure anti-HCV activity, and an MTS assay was used to assess cell viability

KgF25 induces HO-1 mRNA expression and an antiviral IFN response in HCV replicon cells

Also known as heat shock protein 32, HO-1 is an antioxidant enzyme that converts the pro-oxidant heme into carbon

monoxide (CO), free iron and biliverdin. Its transcriptional activation is mediated by the stabilization and binding of nuclear factor-erythroid 2-related factor 2 (Nrf-2) to the HO-1 promoter region. Previous studies have shown that CO and biliverdin are the major mediators of the protective effects of HO-1 in the liver, and anti-inflammatory, anti-apoptotic and anti-oxidative effects have been reported [25, 49, 51]. An effect of HO-1 on HCV replication was demonstrated previously by induction or overexpression of this enzyme [25]. It was also found that HO-1 inhibits HCV replication by activating genes of the interferon signalling pathway such as protein kinase R and oligoadenylate synthetase (OAS) [5, 12, 25, 29, 30, 53]. OAS in particular blocks viral replication by degrading the genomic HCV RNA [47]. HO-1-based therapy options were found to be quite useful in the control of chronic HCV infection [25]. Kg was previously found to possess anti-inflammatory activity [17] and to prevent oxidative damages in primary murine hepatocytes [10, 35, 38, 39] and the HC-O4 human hepatocyte cell line through Nrf-2 activation [36]. In order to find out whether this plant affects antioxidant gene expression in HCV replicon cells, we initially tested the effect of the KgF25 fraction on HO-1 gene transcription in Huh-5-15 cells by qRT-PCR. The relative expression was normalised using the ATP synthase gene as an internal control. The usefulness of this gene as an endogenous control has been validated previously when comparing tumor tissue samples with normal tissue samples [24]. The results showed that KgF25 significantly increased ($p < 0.01$) HO-1 mRNA expression in genotype 1b replicons compared to the DMSO-treated group (Fig. 3a). Moreover, induction of HO-1 was shown to play an important role in suppression of HCV replication through activation of the interferon pathway [30]. Therefore, we also investigated the effect of KgF25 on the antiviral IFN response by measuring OAS gene expression. KgF25 was found to significantly increase OAS-3 and OAS-1 gene transcription by 4.18-fold ($p < 0.001$) and 2.02-fold ($p < 0.01$), respectively, compared to the DMSO control. The induction of these genes was followed by a significant decrease ($p < 0.05$) in the levels of HCV mRNA.

KgF25 downregulates HCV mRNA expression in replicon cells to levels similar to those in cells treated with daclatasvir or telaprevir

Antiviral effects of KgF25 on LucUbiNeo-ET systems were compared to those of the currently approved antivirals daclatasvir (inhibitor of NS5A) and telaprevir (inhibitor of viral protease NS3). Telaprevir was found to suppress HCV replication in a dose-dependent manner (Fig. 3b) with an IC_{50} of 0.84 μM (0.58 μg/mL). A high and significant inhibition ($p < 0.001$) was found with daclatasvir without any dose-dependent activity, suggesting that its IC_{50} value is <0.01 μM (7.39 ng/mL). The values were lower than those found with KgF25 (27.17 μg/mL), meaning that the approved antivirals are more efficient. These results suggest that KgF25 represent an interesting source for the search of anti-HCV compounds. This fraction might target components of the HCV replication complex to inhibit viral replication and could potentially be combined with approved antivirals for hepatitis C treatment.

The inhibition of HCV entry by KgF25 is due to the direct inactivation of free HCVcc particles and does not depend on the genotype tested

To analyse the mechanism of inhibition of viral entry by KgF25, the effect of KgCE and KgF25 were further investigated on free HCVcc virions. These particles were pre-incubated with Kg extract at 25 μg/mL for 1 h and then used to inoculate on Huh-7 cells at 2.5 μg/mL. A significant loss ($p < 0.001$) of virus infectivity was observed in KgCE and KgF25 pre-incubated samples compared to the non-pre-incubated samples, suggesting that KgF25 has a direct effect on free HCVcc particles (Fig. 4a). Similar observations were also made with KgCE.

To investigate whether the inhibition of viral entry by KgF25 is extended to all HCV genotypes, an HCVpp luciferase assay was performed. KgF25 was found to significantly ($p < 0.001$) suppress infectivity of HCVpp 1b, 2a, 3a, 4 and 6 in Huh-7 cells, with at least 95 % reduction at the tested concentration (25 μg/mL) (Fig. 4b). In addition, a significant ($p < 0.001$) reduction in the amount of VSV-Gpp (used as a control virus) entry was also observed. Therefore, inhibition of HCV entry by KgF25 does not seem to be genotype-specific.

Phytochemical compounds and fingerprinting of the KgF25 fraction

Through extensive GC/MS analysis, three compounds were identified in the KgF25 fraction by comparing their retention times and mass spectra with those of the authentic compounds from the NIST library. The retention times of compounds 1, 2 and 3 were 612, 832 and 878 min respectively (Fig. 5). The deprotonated molecules at *m/z* 226.32 indicated a molecular formula of $C_{16}H_{18}O_1$ corresponding to benzene, 1,1′-(oxydiethylidene)bis for compound 1 (PubChem CID: 62342). The deprotonated molecules at *m/z* 255.32 indicated a molecular formula of $C_{16}H_{17}N_1O_2$ corresponding to carbamic acid, (4-methylphenyl)-, 1-phenyl for compound 2. The deprotonated molecules at *m/z* 280 indicated a molecular formula of $C_{20}H_{24}O_1$ corresponding to 6-phenyl, 4-(1′-oxyethylphenyl) hexene for compound 3.

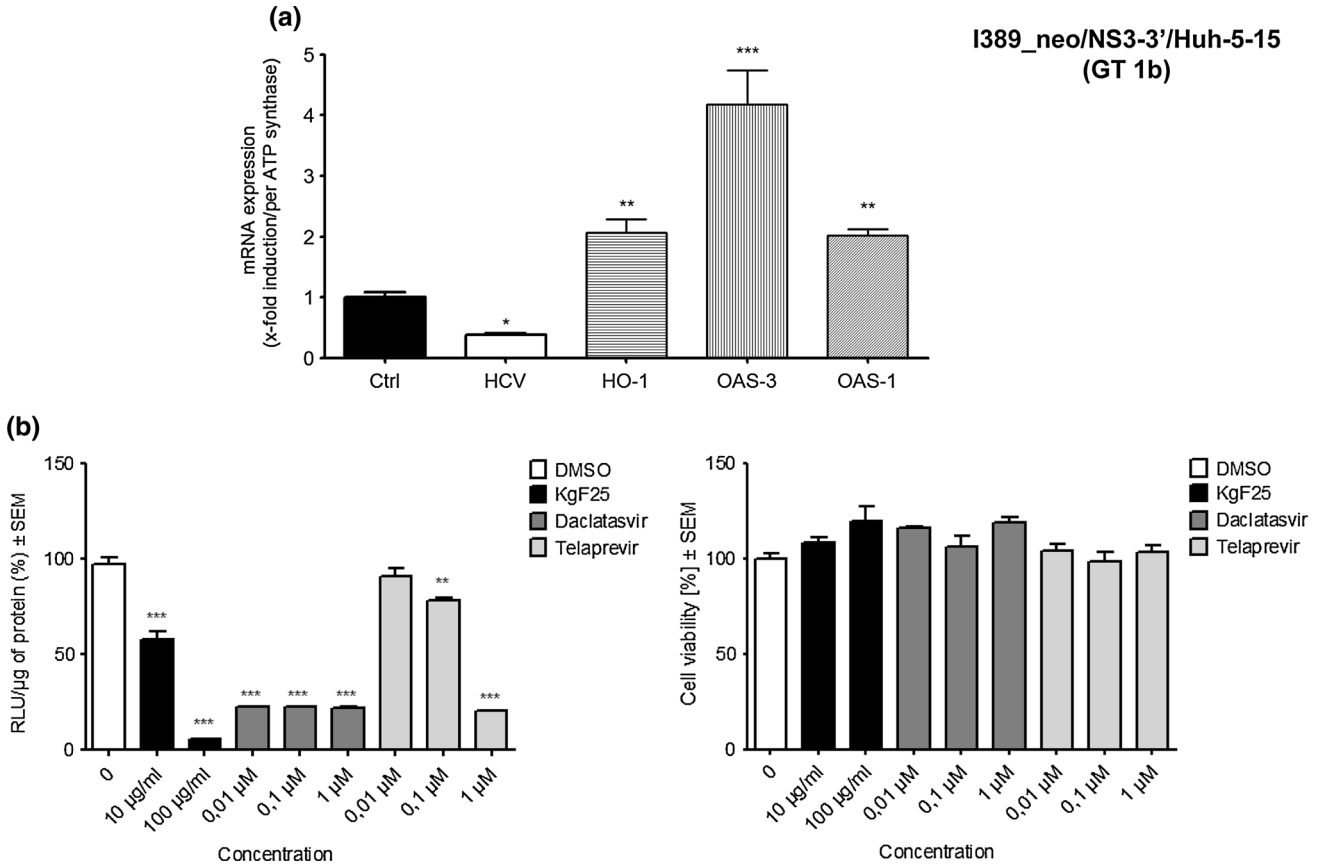

Fig. 3 KgF25 modulates expression levels of HCV mRNA and genes involved in the host response in replicon cells. (**a**) Huh-5-15 cells harboring a type 1b subgenomic HCV RNA without a luciferase gene were treated with the KgF25 extract at 500 µg/mL for 24 h. Cells were lysed, and total RNA was isolated using the TRIzol method. cDNAs were synthesized and used for gene expression analysis by qRT-PCR using SYBR Green. The primers used are listed in Table 1. *, $p < 0.05$; **, $p < 0.01$, ***p<0.001 vs. control (Ctrl) using the Dunnett test. (**b**) LucUbiNeo-ET cells were treated with KgF25 (10-100 µg/mL) or various concentrations (0.1-1 µM) of telaprevir (molecular mass: 679.9 g/mol) or daclatasvir (molecular mass: 738.89 g/mol) for 24 h. The effect on viral replication was assessed by measuring luciferase reporter activity expressed as relative luciferase (RLU/µg of protein). Cell viability was assessed by MTT assay. **, $p < 0.01$; ***, $p < 0.001$ vs. the DMSO group using the Bonferroni test

Discussion

The aim of this work was to investigate the antiviral properties of *K. grandifoliola* against HCV *in vitro.* Through the use of HCVcc and HCV subgenomic reporter constructs, this study provides, for the first time, evidence that both entry and replication steps are targeted by the active fraction from this plant.

Recent studies have suggested that plant extracts from members of the family Meliaceae have antiviral effects against HCV *in vitro*. Wu *et al.* reported the effect of 3-hydroxycarulignan, a compound isolated from *Swietenia macrophylla* against HCV replication using an HCV replicon system [52]. Wahyuni *et al.* demonstrated the effect of crude methanol extracts from *Toonia sureli* against HCVcc-infected cells [50]. However, to the best of our knowledge, little is known about the antiviral effect of plants of the genus *Khaya*, although previous studies on Kg reported many biological activities.

The present study makes a contribution towards addressing this issue, and the results showed an effect of Kg on HCV replication *in vitro* with increased expression of HO-1 and OAS mRNAs in replicon cells. This suggests that induction of HO-1 and IFN-stimulated genes could be an important mechanism by which the KgF25 fraction led to viral inhibition. HO-1 is a negative regulator of inflammation and oxidative stress in the liver [49, 51]. It is involved in the suppression of pro-inflammatory cytokines, and its upregulation is correlated with the inhibition of nitric oxide synthesis. Moreover, the upregulation of the IFN pathway at the protein and mRNA levels has been shown to be highly correlated with the inhibition of viral replication by flavonoids from plant extracts in replicon cells [52]. Chen *et al.* identified lucidone, a substance

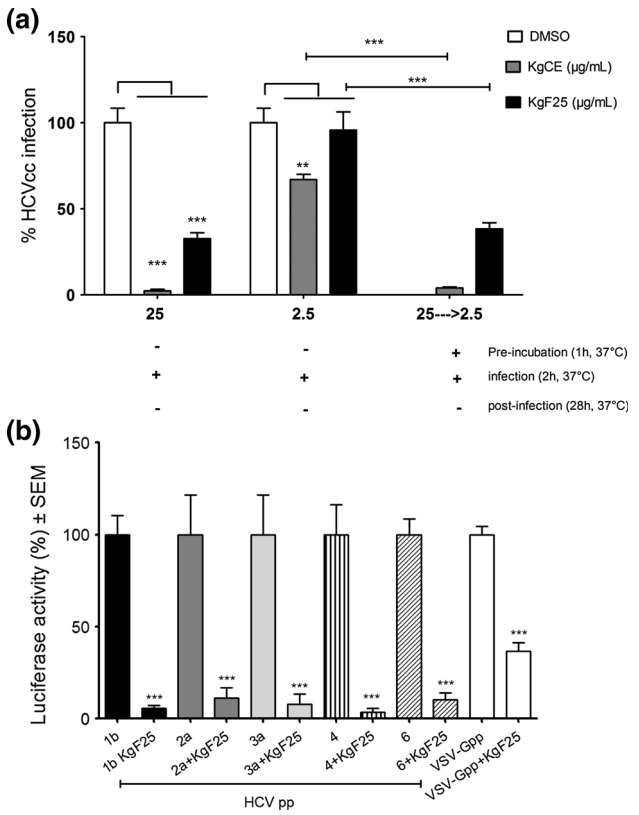

Fig. 4 Effect of KgF25 on free viral particles and the infectivity of HCVpp. (**a**) HCVcc free particles were pre-incubated for 1 h at 37 °C with Kg crude extract (KgCE) or the KgF25 fraction at 25 µg/mL. The viral medium was then diluted tenfold applied to Huh-7 cells for 2 h. Infection rates were evaluated by indirect immunofluorescence. **, $p < 0.01$; ***, $p < 0.001$. (**b**) Huh-7 cells were inoculated with HCVpp (1b, 2a, 3a, 4 and 6) in the presence of KgF25 (25 µg/mL). Infectivity was estimated by luciferase activity and expressed as a percentage compared to the untreated group (DMSO control). *, $p < 0.05$; **, $p < 0.01$ vs. the untreated control using the Bonferroni test

isolated from *Lindera erythrocarpa* as being capable of inducing HO-1 expression and an antiviral IFN response in Ava-5 cells [13]. The same observations were also made by Lee *et al.* who reported an interrelation between the two genes. In addition, the authors mentioned that this activation was under the control of the p38 mitogen activated phosphorylated kinase (MAPK)/Nrf2 pathway [29]. A recent study from our research group demonstrated the ability of this fraction to induce the Nrf-2 pathway in HC-04 cells [37]. Many other signaling pathways might be also involved in the antiviral activity of this fraction. The effect of KgF25 on subgenomic RNA replication suggests interference with the activity or expression of nonstructural proteins of HCV, since this fraction was found to exert antiviral effects comparable to those of daclatasvir and telaprevir. Although the antiviral mechanism needs to be further investigated, KgF25 was found to downregulate HCV RNA expression both at the subgenomic and genomic level; and this effect observed could be due to inhibition of NS3/4A and NS5A, which are particularly important in HCV replication. Moreover, phytochemical analysis of this bioactive fraction enabled identification of three new compounds that might be of importance in the antiviral activity of this plant extract. To the best of our knowledge, this is the first time that such compounds have been isolated from *K. grandifoliola*. In addition, only benzene, 1,1′-(oxydiethylidene)bis (1) (PubChem CID: 62342) has been screened for biological activity, including cytotoxic activity. However, no information is available on the activity of other compounds. Most of the HCV inhibitors developed to date inhibit downstream viral replication, through blockage of the viral NS3/4A protease or NS5B polymerase and its cofactor NS5A. Therefore, KgF25 may be a source for the isolation of new plant-derived antivirals that act through the aforementioned mechanisms. It would be interesting for further studies to analyse the signalling pathways involved in the antiviral activity of KgF25 and to isolate its active molecules.

Key steps of the HCV life cycle include entry, uncoating of the viral genome, viral protein synthesis, viral genome replication, and assembly and secretion of virions. To confirm the effect of KgCE and KgF25 in an *in vitro* model allowing the complete cycle of HCV to occur, these extracts were tested on HCVcc-infected cells. KgCE and KgF25 treatment strongly decreased HCV infection, and particularly viral entry when compared with the vehicle control (DMSO), while the replication step was moderately reduced (Fig. 2a and b). Most importantly, when HCV infection remained low (<10 %) in the KgCE- and KgF25-treated groups, cell viability remained nearly 100 % (data not shown). This implies that the profound antiviral activity of these compounds at 25 µg/mL is not attributable to cytotoxicity. However, KgCE was found to be more efficient than KgF25, with selectivity indices of 70 and 16, respectively. Our results also revealed that KgF25 targets early steps of HCV entry, specifically by inactivating cell-free virions, abolishing subsequent infection (Fig. 4a). Using the HCVpp system, we found a pan-genotypic activity of KgF25 on viral entry (Fig. 4b), which means that the sensitivity of HCV to this plant fraction does not depend on the genotype tested. Therefore, it is possible that KgF25 acts on the same target for entry inhibition. Since the extract from neem (*Azadirachta indica*, a plant from the same family as Kg) also exerted antiviral effect on herpes simplex virus 1 (an enveloped virus) through action on its glycoproteins [22], we speculate that compounds present in KgCE and KgF25 could possibly target the HCV glycoproteins to structurally render the viral particle inactive or too bulky, or to impede its ability to bind to the host-cell receptor [11].

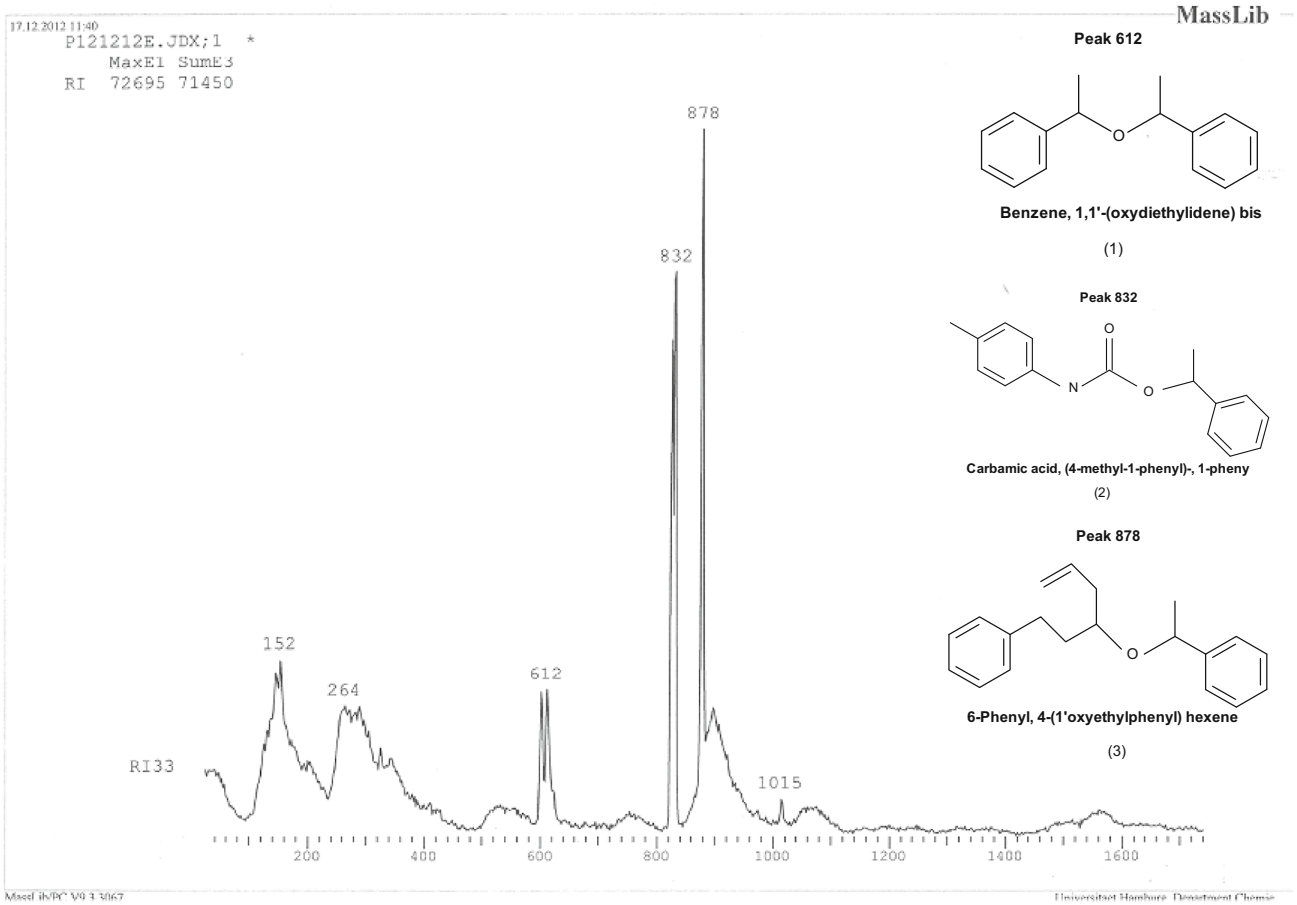

Fig. 5 GC-chromatogram of KgF25 and structure of some chemical constituents identified by mass spectrometry

In conclusion, this study demonstrates that *K. grandifoliola* inhibits HCV infection by inhibiting different steps of the viral cycle, primarily the entry step. This inhibition includes the inactivation of free HCVcc virions with pan-genotypic activity and the transcriptional activation of some cellular genes such as HO-1 and OAS. The anti-HCV activity of this plant could be attributed to the presence of active compounds in the KgF25 fraction, but efforts are still needed to identify these compounds. Moreover, these data provide a new impetus to sustain the traditional use of *K. grandifoliola* against viral hepatitis and maybe to fully explore its clinical utility for the treatment of hepatitis C in Cameroon.

Acknowledgments We thank the DAAD (German Academic Exchange Service) who Granted a six-month scholarship to B.R.T.G. at the University of Hamburg in Germany (Grant Number A/12/90499). We also thank Professor Hartmut Schlüter from the Department of Clinical Chemistry of the Hamburg University Medical Center for the GC and mass spectrometry analysis. This work was partly funded by the 'Agence Nationale de la Recherche sur le Sida et les Hépatites virales' (ANRS) in France through the "'Centre d'Infection et d'Immunité de Lille"' and by the International Foundation for Sciences (IFS), Stockholm, Sweden, who supported the collection and fractionation of plant material through Grant No. F/4223-2, awarded to Dr. F.N. Njayou. Finally, we are grateful to Dr. Manfo Faustin Pascal for the critical review of this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Adeyemi A, Gbilade A (2006) Antianaemic activity of Spondias mombin and Khaya grandifoliola aqueous extracts on rats. J Pharmacy Biores 3:94–97
2. Andoulo FA, Noah DN, Djapa R, Kowo M, Talla P, Medjo EH, Djomkam IK, Nonga BN, Njoya O, Ndam ECN (2014) Epidemiology of hepatitis C: related hepatocellular carcinoma in Cameroon. Pan Afr Med J. doi:10.11604/pamj.2014.19.379.4143.eCollection
3. Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, Scarselli E, Cortese R, Nicosia A, Cosset FL (2003) Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 278:41624–41630. doi:10.1074/jbc

4. Bickii J, Njifutie N, Foyere JA, Basco LK, Ringwald P (2000) In vitro antimalarial activity of limonoids from Khaya grandifoliola C.D.C. (Meliaceae). J Ethnopharmacol 69:27–33
5. Bonifaz V, Shan Y, Lambrecht RW, Donohue SE, Moschenross D, Bonkovsky HL (2009) Effects of silymarin on hepatitis C virus and haem oxygenase-1 gene expression in human hepatoma cells. Liver Int 29:366–373. doi:10.1111/j.1478-3231.2008.01833.x
6. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
7. Bumah VV, Essien EU, Agbedahunsi MJ, Ekah OU (2005) Effect of *Khaya grandifoliola* (Meliaceae) on some biochemical parameters in rats. J Ethnopharmacol 102:446–449. doi:10.1016/j.jep.2005.07.014
8. Bumah V, Agbedahunsi J (2010) Toxicological studies of the stem bark extract of *Khaya grandifoliola* in rats. Niger J Nat Prod Med 13:46–52. doi:10.4314/njnpm.v13i1.61601
9. CADTH Rapid Response reports (2014) Triple therapy for Hepatitis C in previous non-responders: a review of the clinical effectiveness and safety [Internet]. Canadian Agency for Drugs and Technologies in Health, Ottawa (ON), pp 1–15
10. 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. doi:10.1002/hep.24803
11. Calland N, Sahuc M-E, Belouzard S, Pène V, Bonnafous P, Mesalam AA, Deloison G, Descamps V, Sahpaz S, Wychowski C, Lambert O, Brodin P, Duverlie G, Meuleman P, Rosenberg AR, Dubuisson J, Rouillé Y, Séron K (2015) Polyphenols inhibit hepatitis C virus entry by a new mechanism of action. J Virol 89:1–11. doi:10.1128/JVI.01473-15
12. Chen M, Lee M, Chuang J, Li Y, Ning S, Chen J, Liu Y (2012) Curcumin inhibits HCV replication by induction of heme oxygenase-1 and suppression of AKT. Int J Mol Med 30:1021–1028. doi:10.3892/ijmm.2012.1096
13. Chen WC, Wang SY, Chiu CC, Tseng CK, Lin CK, Wang HC, Lee JC (2012) Lucidone suppresses hepatitis C virus replication by Nrf2-mediated heme oxygenase-1 induction. Antimicrobial Agents Chemother. doi:10.1128/AAC.02053-12
14. 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. doi:10.1002/hep.24610
15. Delgrange D, Pillez A, Castelain S, Cocquerel L, Rouillé Y, Dubuisson J, Wakita T, Duverlie G, Wychowski C (2007) Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. J Gen Virol 88:2495–2503. doi:10.1099/vir.0.82872-0
16. Dubuisson J, Hsu HH, Cheung RC, Greenberg HB, Russell DG, Rice CM (1994) Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J Virol 68:6147–6160
17. Falodun A, Poh CF, Adelusi SA, Chemistry P, Pharmacy FOF, Of F, Medical B, Of C, Sciences H, Island W (2009) Phytochemical and anti-inflammatory evaluation of khaya grandifoliola stem bark extract. Int J PharmTech Res 1:1061–1064
18. Goueslain L, Alsaleh K, Horellou P, Roingeard P, Descamps V, Duverlie G, Ciczora Y, Wychowski C, Dubuisson J, Rouillé Y (2010) Identification of GBF1 as a cellular factor required for hepatitis C virus RNA replication. J Virol 84:773–787. doi:10.1128/JVI.01190-09
19. Haid S, Novodomská A, Gentzsch J, Grethe C, Geuenich S, Bankwitz D, Chhatwal P, Jannack B, Hennebelle T, Bailleul F, Keppler O, Poenisch M, Bartenschlager R, Hernandez C, Lemasson M, Rosenberg A, 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. doi:10.1053/j.gastro.2012.03.036 **(Epub 2012 Mar 27)**
20. Hashem FA, Aboutabl EA, El-souda SS, Selim A, Shaker K, Maamoun AA (2014) Composition of lipoidal matter and evaluation of hepatoprotective, cytotoxic, and antioxidant activities of *Khaya grandifoliola* C. DC. growing in Egypt. Egypt Pharm J 13:13–20. doi:10.4103/1687-4315.135576
21. Hashem FA, Aboutabl EA, El Souda SS, Moharam M, Maamoum AA, Shabana M (2013) Desmutagenic and antimutagenic potential of phenolics from *Khaya grandifoliola* (C.DC.) Meliaceae. Egypt Pharm J 12:148–154. doi:10.4103/1687-4315.124018
22. Hsu W-C, Chang S-P, Lin L-C, Li C-L, Richardson CD, Lin C-C, Lin L-T (2015) Limonium sinense and gallic acid suppress hepatitis C virus infection by blocking early viral entry. Antiviral Res 118:139–147. doi:10.1016/j.antiviral.2015.04.003
23. Izquierdo-García E, Escobar-Rodríguez I (2012) Systematic review of new protease inhibitors interactions: telaprevir and boceprevir. Farm Hosp 36:469–482. doi:10.7399/FH.2012.36.6.47
24. Janssens N, Janicot M, Perera T, Bakker A (2004) Housekeeping genes as internal standards in cancer research. Mol Diagn 8:107–113
25. Kah J (2014) Characterization of heme oxygenase 1 based therapy options in chronic persistent HCV infection. University of Hamburg
26. Krieger N, Lohmann V, Bartenschlager R (2001) Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J Virol 75:4614–4624. doi:10.1128/JVI.75.10.4614-4624.2001
27. Lan K-H, Wang Y-W, Lee W-P, Lan K-L, Tseng S-H, Hung L-R, Yen S-H, Lin H-C, Lee S-D (2012) Multiple effects of Honokiol on the life cycle of hepatitis C virus. Liver Int 32:989–997. doi:10.1111/j.1478-3231.2011.02621.x **(Epub 2011 Aug 11)**
28. Lavanchy D (2011) Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect 17:107–115. doi:10.1111/j.1469-0691.2010.03432
29. Lee JC, Tseng CK, Young KC, Sun HY, Wang SW, Chen WC, Lin CK, Wu YH (2014) Andrographolide exerts anti-hepatitis C virus activity by upregulating heme oxygenase-1 via the p38 MAPK/Nrf2 pathway in human hepatoma cells. Br J Pharmacol 171:237–252. doi:10.1111/bph.12440
30. Lehmann E, El-Tantawy WH, Ocker M, Bartenschlager R, Lohmann V, Hashemolhosseini S, Tiegs G, Sass G (2010) The heme oxygenase 1 product biliverdin interferes with hepatitis C virus replication by increasing antiviral interferon response. Hepatology 51:398–404. doi:10.1002/hep.23339
31. Liu S, Chen R, Hagedorn CH (2015) Tannic acid inhibits hepatitis C virus entry into Huh7.5 cells. PLoS One 10:e0131358. doi:10.1371/journal.pone.0131358
32. Lohmann V, Korner F, Koch J, Herian U, Theilmann LBR (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110–113
33. Makinde JM, Awe SO, Agbedahunsi JM (2006) Effect of *Khaya grandifoliola* extract on *Plasmodium berghei* berghei in mice. Phytother Res 2:30–32. doi:10.1002/ptr.2650020104
34. Makki A (2014) Toxicity of *Khaya grandifoliola* (African mahogany) bark extract against biomphalaria snails. In: Journal of Biomedical and Pharmaceutical Research. http://jbpr.in/index.php/jbpr/article/view/344/436. Accessed 24 May 2015
35. Njayou F, Moundipa P, Tchana N, Ngadjui T, Tchouanguep F (2008) Inhibition of microsomal lipid peroxidation and protein oxidation by extracts from plants used in Bamun folk medicine

(Cameroon) against hepatitis. Afr J Tradit Complement Altern Med 5:278–289
36. Njayou FN, Amougou AM, Fouemene Tsayem R, Njikam Manjia J, Rudraiah S, Bradley B, Manautou JE, Fewou Moundipa P (2015) Antioxidant fractions of *Khaya grandifoliola* C.DC. and *Entada africana* Guill. et Perr. induce nuclear translocation of Nrf2 in HC-04 cells. Cell Stress Chaperones. doi:10.1007/s12192-015-0628-6
37. Njayou FN, Amougou AM, Fouemene Tsayem R, Njikam Manjia J, Rudraiah S, Bradley B, Manautou JE, Fewou Moundipa P (2015) Antioxidant fractions of *Khaya grandifoliola* C.DC. and *Entada africana* Guill. et Perr. induce nuclear translocation of Nrf2 in HC-04 cells. Cell Stress Chaperones 20:991–1000. doi:10.1007/s12192-015-0628-6
38. Njayou FN, Galani TBR, Ngadjui TB, Moundipa FP (2013) Hepatoprotective activity of three Cameroonian medicinal plants against oxidative damages induced by paracetamol and CCl4 in primary rat hepatocytes. Int J Pharmacy Photon 104:306–314
39. Njayou NF, Moundipa PF, Tchana NA, Tchouanguep F (2004) Antihepatotoxic potential of three Bamun folk medicinal plants. Cameroonian J Biochem Sci 4:325–330
40. Njifutie N, Njikam N (2006) Curative dose of *K. grandifoliola* stem bark for the treatment of gastric ulcers using Wistar rats. Pharm Biol 44:152–155
41. Njoya O, Ntchama L, Tagni M, Dang I, Kowo M (2013) Relapses in the treatment of genotype 2 viral hepatitis C, a cause of concern in the blacks. Indian J Gastroenterol 33:292. doi:10.1007/s12664-013-0385-3
42. OMS (2012) Hépatite C. Aide-mémoire 164:2–5
43. Onifade AK (2006) Effect of essential oils of some Meliaceaous plants on aflatoxin production and growth of *Aspergillus parasiticus*. J Food Technol 4:322–324
44. Polyak SJ, Oberlies NH, Pécheur E-I, Dahari H, Ferenci P, Pawlotsky J-M (2013) Silymarin for HCV infection. Antiviral Therapy 18:141–147. doi:10.3851/IMP2402
45. Rouillé Y, Helle F, Delgrange D, Roingeard P, Voisset C, Blanchard E, Belouzard S, McKeating J, Patel AH, Maertens G, Wakita T, Wychowski C, Dubuisson J (2006) Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. J Virol 80:2832–2841. doi:10.1128/JVI.80.6.2832-2841.2006
46. Sarrazin C, Zeuzem S (2010) Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology 138:447–462. doi:10.1053/j.gastro.2009.11.055
47. Sen GC, Sarkar SN (2007) The interferon-stimulated genes : targets of direct signaling by interferons, double-stranded RNA, and viruses. Curr Topics Microbiol Immunol 316:233–250
48. Stephen UA, Abiodun F, Osahon A, Ewaen E (2009) Phytochemical analysis and antibacterial activity of *Khaya grandifoliola* stem bark. J Biol Sci 9:63–67. doi:10.3923/jbs.2009.63.67
49. Tang J, Li L, Li C-M, Wu J, Sun Y, Wang G-L (2015) Up-regulation of HO-1 with haemin alleviates LPS-stimulated pro-inflammatory responses through down-regulation of p38 signaling pathways in rat liver. Scand J Immunol. doi:10.1111/sji.12352
50. Wahyuni T, Tumewu L, Permanasari A, Apriani E, Adianti M, Rahman A, Widyawaruyanti A, Lusida M, Fuad A, Soetjipto N, Nasronudin N, Fuchino H, Kawahara N, Shoji I, Deng L, Aoki C, Hotta H (2013) Antiviral activities of Indonesian medicinal plants in the East Java region against hepatitis C virus. Virol J 10:259. doi:10.1186/1743-422X-10-259
51. Wei J, Fan G, Zhao H, Li J (2015) Heme oxygenase-1 attenuates inflammation and oxidative damage in a rat model of smoke-induced emphysema. Int J Mol Med. doi:10.3892/ijmm.2015.2353
52. Wu SF, Lin CK, Chuang YS, Chang FR, Tseng CK, Wu YC, Lee JC (2012) Anti-hepatitis C virus activity of 3-hydroxy caruilignan C from *Swietenia macrophylla* stems. J Viral Hepatitis 19:364–370. doi:10.1111/j.1365-2893.2011.01558.x **(Epub 2011 Nov 24)**
53. Wuestenberg A, Kah J, Singethan K, Sirma H, Keller AD, Rosal SRP, Schrader J, Loscher C, Volz T, Bartenschlager R, Lohmann V, Protzer U, Dandri M, Lohse AW, Tiegs G, Sass G (2014) Matrix conditions and KLF2-dependent induction of heme oxygenase-1 modulate inhibition of HCV replication by fluvastatin. PloS One 9:e96533. doi:10.1371/journal.pone.0096533
54. Yuan T, Zhu R, Zhang H, Odeku OA, Yang S, Liao S, Yue J (2010) Structure determination of grandifotane A from *Khaya grandifoliola* by NMR, X-ray diffraction, and ECD calculation. Org Lett 12:252–255. doi:10.1021/ol902565s