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# An ethanol extract of *Lysimachia mauritiana* exhibits inhibitory activity against hepatitis E virus genotype 3 replication

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**Hepatitis E virus (HEV) is an etiological agent of acute hepatitis E, a self-limiting disease prevalent in developing countries. HEV can cause fulminant hepatic failure with high mortality rates in pregnant women, and genotype 3 is reported to trigger chronic hepatitis in immunocompromised individuals worldwide. Screening of plant extracts for compounds with potential anti-HEV effects led to the identification of a 70% ethanol extract of *Lysimachia mauritiana* (LME) that interferes with replication of the swine HEV genotype 3 replicon. Furthermore, LME significantly inhibited replication of HEV genotype 3 and expression of HEV ORF2 in infected cells without exerting cytotoxic effects. Collectively, our findings demonstrate the potential utility of LME in the development of novel antiviral drugs against HEV infection.**

***Keywords***: hepatitis E virus, antiviral, *Lysimachia mauritiana*

## Introduction

Hepatitis E virus (HEV) is a small non-enveloped virus with a single-stranded, positive-sense RNA genome pf ~7.2 kb (Tam *et al.*, 1991; Yamada *et al.*, 2009; Meng, 2010). The HEV RNA genome is 5′ capped and 3′ polyadenylated and contains a short 5′ noncoding region (NCR), three open reading frames (ORFs), and 3′ NCR (Tam *et al.*, 1991; Emerson and Purcell, 2003). ORF1 encodes a nonstructural protein with several functional domains required for RNA-dependent RNA polymerase (RdRp) function (Nan and Zhang, 2016). ORF2 encodes the major capsid protein involved in the assembly of HEV particles and immunogenicity (Tang *et al.*, 2011). ORF3 encodes a small multifunctional protein essential for HEV replication and pathogenicity (Kalia *et al.*, 2009; Moin *et al.*, 2009; Chandra *et al.*, 2010; Cao and Meng, 2012; He *et al.*, 2016). Functional interaction between ORF2 and ORF3 are proposed to play an important role in the assembly of HEV, although the exact mechanisms are not fully understood at present (Graff *et al.*, 2006; Huang *et al.*, 2007; Chandra *et al.*, 2010).

HEV is associated with waterborne epidemics (Aggarwal and Naik, 1994; Teshale *et al.*, 2010). The virus is spread via the fecal-oral route and transmitted in drinking water contaminated with feces and undercooked meat products (Kamar *et al.*, 2012; Johne *et al.*, 2014; Van der Poel, 2014). HEV causes acute hepatitis with symptoms of fever, fatigue, vomiting or abdominal pain. Acute hepatitis E is commonly self-limiting and does not usually develop into a chronic condition (Bose *et al.*, 2011; Schlosser *et al.*, 2012). However, the mortality rate of pregnant women with HEV infection is significantly higher (~30%) than that of immunocompetent individuals (~3.3%) (Khuroo *et al.*, 1995; Patra *et al.*, 2007; Begum *et al.*, 2009). Infection with HEV genotype 3 leading to chronic hepatitis has additionally been documented in immunosuppressed individuals, such as liver, kidney and stem-cell transplant recipients (Gérolami *et al.*, 2008; Navaneethan *et al.*, 2008; Schemmerer *et al.*, 2016).

Among the four major genotypes of HEV, genotype 3 is of major concern, since it can be transmitted via zoonosis and cause chronic hepatitis (Gérolami *et al.*, 2008; Navaneethan *et al.*, 2008; Schemmerer *et al.*, 2016). Genotype 3 transmission has been reported in swine, wild boar, deer, mongoose and rodents. Notably, swine HEV strains are closely related to human strains with ~90% sequence homology (Clayson *et al.*, 1995; Meng, 2010; Dell'Amico *et al.*, 2011). Thus, pork meat products contaminated with swine HEV strains can be transmitted to humans via consumption of infected meat including sausages.

At present, no specific treatments are available for HEV infection. While ribavirin and interferon α (IFN-α) are routinely used to treat chronic hepatitis E, long-term administration of these drugs can trigger various side-effects (Pischke *et al.*, 2013; Debing *et al.*, 2014). In addition to these compounds, a vaccine based on HEV genotype 1 has been licensed in China that is reported to function against infections caused by both genotypes 1 and 4 (Zhu *et al.*, 2010; Larralde and Petrik, 2017; Shrestha *et al.*, 2017). The issue of whether this vaccine provides protection against HEV genotype 3 and is effective in patients at risk of chronic HEV is yet to be established (Zhu *et al.*, 2010; Larralde and Petrik, 2017). Furthermore, the vaccine is not approved for pregnant women with HEV infection due to safety concerns. Consequently, the development of a novel antiviral strategy against HEV, in particular, genotype 3, remains an urgent medical need.

Natural products contain several as yet undetermined compounds that may be a potential source of novel anti-

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viral drugs that exert fewer side-effects (Abdelmohsen *et al.*, 2017). Screening of plant extracts for compounds with potential inhibitory effects led to the identification of a 70% ethanol extract of *Lysimachia mauritiana* (LME) that interferes with replication of the swine HEV genotype 3 replicon. The antiviral activity of LME against HEV infection was further investigated in the present study.

## Materials and Methods

### Cells, replicons, viruses and plant materials

The human adenocarcinoma cell line, A549, and human hepatocellular carcinoma cell line, Huh 7.5, were obtained from the Korean Cell Line Bank and maintained as described previously (Cao and Meng, 2012; Johne *et al.*, 2014). A recombinant genotype 3 swine HEV replicon with a *Renilla* luciferase reporter (pSHEV3-luc) was kindly provided by Dr. Xiang-Jin Meng (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) (Cao and Meng, 2012). *In vitro* transcription and transfection of pSHEV3-luc were performed according to a previously documented procedure (Cao and Meng, 2012). The HEV genotype 3 strain 47832c was a kind gift from Dr. Reimar Johne (Federal Institute for Risk Assessment, Berlin, Germany) (Johne *et al.*, 2014). Maintenance and propagation procedures for 47832c have been described previously (Johne *et al.*, 2014). Plant material (*Lysimachia mauritiana* Lam.) was collected from Jeju Island in Republic of Korea, and voucher specimens for the samples deposited at the herbarium of the Department of Biological Sciences at Sungkyunkwan University (specimen number SKK170623001). Extraction of plant materials was performed in accordance with reported procedures (Bae and Song, 2017).

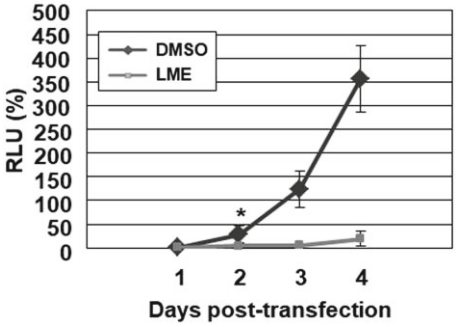


**Fig. 1. LME interferes with pSHEV3-luc replication.** Huh7.5 cells were transfected with RNA transcripts from the pSHEV3-luc replicon and treated with DMSO or LME at a concentration of 10 μg/ml. At 3 days post-treatment, cells were re-treated with either DMSO or LME. Luciferase activities determined at 1, 2, 3, and 4 days post-transfection. *Renilla* luciferase activity of the pSHEV3-luc replicon was normalized with constitutive firefly luciferase activity of luc-pcDNA3 transcripts and expressed as RLU. To calculate relative luciferase activity, that of the pSHEV3-luc replicon in the presence of DMSO at 1 day post-transfection was set as 1. Data represent means ± standard deviations (SD) of at least three independent experiments. Significant differences between samples were determined based on *P* values obtained from the Student's *t*-test (* $P < 0.05$). RLU, relative luciferase light unit.

### Cell viability and luciferase reporter assays

CellTiter-Glo Luminescent Cell Viability Assay to determine the ATP levels in metabolically active cells and Dual-Luciferase Reporter Assay were conducted in keeping with the manufacturer's instructions (Promega).

### Quantification of HEV RNA

HEV RNA levels were determined using quantitative reverse transcription PCR (qRT-PCR). Total RNA was extracted using HiGene Total RNA Prep kit (BIOFACT) and reverse-transcribed into complementary DNA (cDNA) with the aid of a TOPscrip™ cDNA Synthesis Kit (Enzynomics) according to the manufacturers' instructions. HEV cDNA was quantified in a StepOnePlus Real-Time PCR system (Applied Biosystems) using HOT FIREPol EvaGreen quantitative PCR (qPCR) mix Plus (Solis BioDyne) and the following primers: HEV ORF2, 5′-TATCGGGTTGTCCGAGCTAC-3′ and 5′-TGCCGGGTTGAACTAGAATC-3′; GAPDH, 5′-CATGAGAAGTATGACAACAGCCT-3′ and 5′-AGTCCTTCCACGATACCAAAGT-3′.

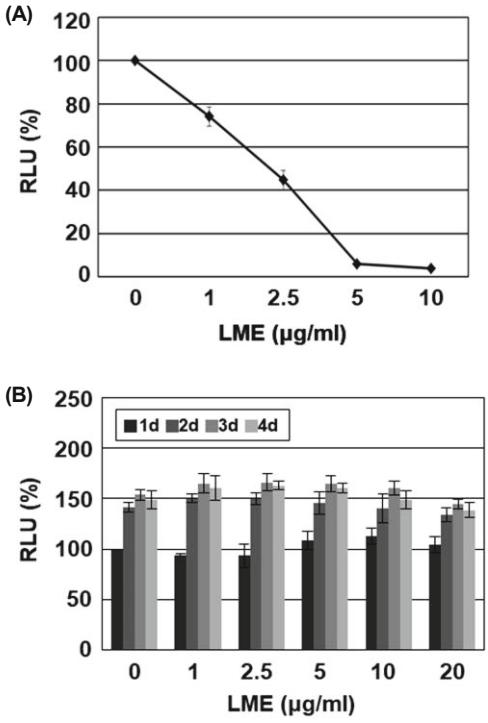


**Fig. 2. LME inhibits pSHEV3-luc replication in a dose-dependent manner.** (A) Huh7.5 cells transfected with RNA transcripts from the pSHEV3-luc replicon were treated with LME at concentrations of 0, 1, 2.5, 5, or 10 μg/ml. Cells were re-treated with either DMSO or LME at 3 days post-transfection, and luciferase activities determined at 4 days post-transfection. To calculate relative luciferase activity, that of the pSHEV3-luc replicon in cells treated with LME (0 μg/ml) was set as 100%. Data represent means ± SD of at least three independent experiments. $IC_{50}$ values were calculated using GraphPad Prism 7 software. (B) Huh7.5 cells were treated with LME at concentrations of 0, 1, 2.5, 5, 10, or 20 μg/ml, and re-treated with LME at 3 days post-transfection. Cell viability was determined at 1, 2, 3, and 4 days after treatment using the CellTiter-Glo Luminescent cell viability assay. To calculate relative luciferase activity, RLU of cells at 1 day post-treatment in the 0 μg/ml LME group was set as 100%. Data represent means ± SD of at least three independent experiments.

### Immunofluorescence assay

The immunofluorescence assay (IFA) was performed as described previously (Huang *et al.*, 2005). Briefly, at 14 days post-infection, A549 cells were fixed with 80% acetone for 20 min at -20°C and stained with mouse-HEV ORF2 antibody (MAB8002) (Millipore) and fluorochrome-conjugated secondary anti-mouse IgG antibody (4408) (Cell signaling). Fluorescence was detected, and images analyzed using an inverted Nikon TS100-F fluorescence microscope equipped with a digital camera and Nikon NIS-Elements microscope imaging software.

## Results

### Effects of LME on replication of HEV genotype 3 replicon

To investigate the antiviral effects of LME against HEV, Huh7.5 cells were transfected with RNA transcripts from pSHEV3-luc and treated with DMSO or LME (10 μg/ml). Replication of the pSHEV3-luc replicon was determined by measuring luciferase activities at 1, 2, 3, and 4 days post-transfection (Fig. 1). In DMSO-treated cells, luciferase activity of the pSHEV3-luc replicon at 4 days post-transfection was increased to 357.2-fold, compared to that at 1 day post-transfection. Relative to DMSO-treated cells, luciferase activity transfected cells treated with LME was significantly suppressed to 4.7% and 5.2% at 3 and 4 days post-transfection, respectively (Fig. 1). Our results clearly demonstrate that LME exerts potent inhibitory activity against replication of HEV genotype 3 replicon.

### LME suppresses HEV genotype 3 replication in a concentration-dependent manner

To determine the half maximal inhibitory concentration ($IC_{50}$) value of LME against pSHEV3-luc replication, Huh7.5 cells transfected with RNA transcripts from the pSHEV3-luc replicon were treated with LME (0, 1, 2.5, 5, or 10 μg/ml), and luciferase activities determined at 4 days post-transfection (Fig. 2A). LME suppressed the luciferase activities of pSHEV-luc replicon in a concentration-dependent manner with an $IC_{50}$ value of 2.141 ± 0.242 μg/ml.

To further examine whether the inhibitory effects of LME are associated with cytotoxicity, the influence of LME on cell viability was further determined. Huh7.5 cells were treated with LME (0, 1, 2.5, 5, 10, or 20 μg/ml), and the intracellular ATP levels indicative of metabolically active cells analyzed at 1, 2, 3, and 4 days after treatment (Fig. 2B). Notably, LME did not exert adverse effects on Huh7.5 cell viability, indicating that its inhibitory effects on luciferase activity was not mediated through induction of cytotoxicity.

### LME interferes with replication of HEV genotype 3

In view of the finding that LME suppresses the luciferase activity of pSHEV3-luc without affecting the viability of Huh7.5 cells, its antiviral effects against HEV genotype 3 strain were further investigated (Fig. 3). To this end, A549 cells were infected with the HEV genotype 3 strain 47832c and subsequently treated with either DMSO or LME (10 μg/ml). At 1, 3, 7, and 14 days post-infection, the levels of HEV RNA were determined via qRT-PCR (Fig. 3A). In DMSO-treated cells, HEV RNA levels were increased 4.5- and 6.7-fold at 7

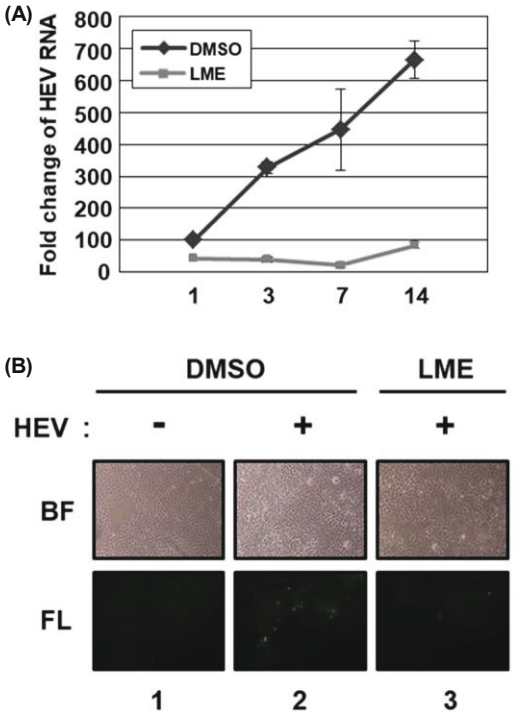


**Fig. 3. LME inhibits HEV genotype 3 replication.** A549 cells were infected with HEV genotype 3 strain 47832c and treated with either DMSO or LME at a concentration of 10 μg/ml. (A) At 1, 3, 7, and 14 days post-infection, the relative amounts of HEV RNA were determined via qRT-PCR as described in Materials and Methods. Data represent the average of three independent experiments. To determine the relative differences between samples, HEV RNA amounts in HEV-infected cells 1 day after treatment in the 0 μg/ml LME group was set at 100. Data represent means ± SD of at least three independent experiments. (B) At 14 days post-infection, expression of the HEV ORF2 capsid protein in A549 cells was evaluated using immunofluorescence microscopy. BF, Bright field microscopic image; FL, Fluorescence microscopic image.

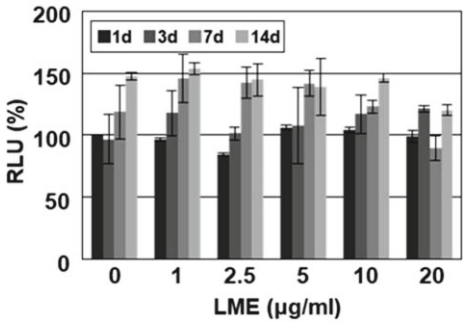


**Fig. 4. LME exerts no adverse effects on A549 cells.** A549 cells were treated with varying concentrations of LME (0, 1, 2.5, 5, 10, or 20 μg/ml). Cells were re-treated with LME every 3 days after initial treatment, and cell viability determined at 1, 3, 7, and 14 days after treatment using the CellTiter-Glo Luminescent cell viability assay. To calculate relative luciferase activity, RLU of cells 1 day after treatment in the 0 μg/ml LME group was set as 100%. Data represent means ± SD of at least three independent experiments.

and 14 days post-infection, respectively (Fig. 3A). On the other hand, the amounts of HEV RNA in LME-treated cells were significantly reduced to 4.4% and 11.9% relative to DMSO-treated cells at 7 and 14 days post-infection, respectively (Fig. 3A). In addition, expression of ORF2 capsid protein in A549 cells infected with 47832c was significantly down-regulated at 14 days post-infection (Fig. 3B).

To further establish the effects of LME on cell viability, A549 cells were treated with LME (0, 1, 2.5, 5, 10, or 20 μg/ml), and intracellular ATP levels analyzed at 1, 3, 7, and 14 days after treatment (Fig. 4). Consistent with data obtained with Huh7.5 cells, LME did not exert cytotoxic effects on A549 cells up to a concentration of 10 μg/ml (Fig. 4). Taken together, our results indicate that LME contains a bioactive constituent(s) that interferes with HEV replication.

## Discussion

Since HEV infection is detrimental to pregnant women, and genotype 3 HEV can induce chronic infection in immunocompromised patients, there is an increasing demand for effective HEV-specific antiviral drugs with low side-effects and high efficacy (Khuroo *et al.*, 1995; Patra *et al.*, 2007; Begum *et al.*, 2009; Bose *et al.*, 2011; Schlosser *et al.*, 2012). Data from the current study demonstrate that LME possesses inhibitory activity against replication of genotype 3 HEV without accompanying cytotoxic effects.

The biological activities of LME have not been extensively characterized to date. Previously, we reported that LME inhibits replication of laboratory and clinical strains of varicella-zoster virus (VZV) by suppressing expression of the viral transactivator, immediate-early 62 protein (IE62) (Bae and Song, 2017). The antiviral activities of LME against both VZV and HEV make it a valuable source of potential antiviral drug candidates.

The specific chemical components of LME that exert antiviral activities against VZV and/or HEV remain to be determined. To date, salicylic acid and flavonol glycosides have been characterized from species of the genus *L. mauritiana* (Yasukawa *et al.*, 1990; Yasukawa and Takido, 1993), but the majority of chemical components remain to be identified. LME is speculated to contain different bioactive compounds specifically targeting VZV or HEV or a single bioactive compound with universal antiviral activity. In this case, LME may affect the cellular system essential for viral replication. Further research is warranted to determine the bioactive components of LME and their antiviral mechanisms of action.

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