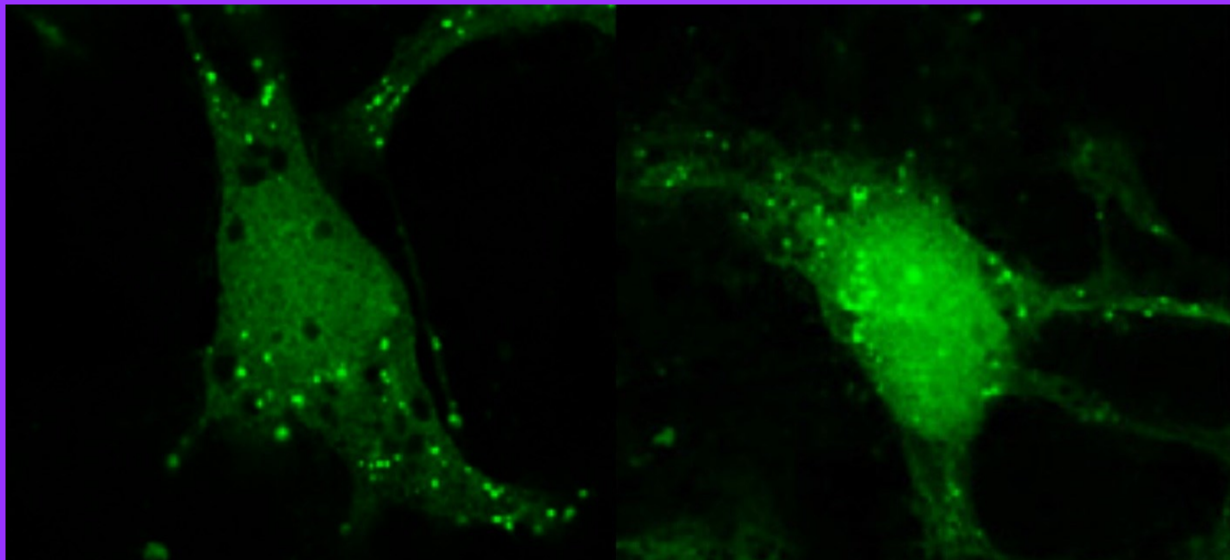

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Nirtetralin inhibits growth of hepatitis E virus in HepaRG™ cells

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Abstract

Among numerous established in human hepatoma cell lines, none has been shown susceptible to hepatitis E virus (HEV) infection. Differentiation and infect ability are maintained but when these cells are cultured in the presence of corticoids and dimethyl sulfoxide. On exposure to the nirtetralin, the virion particles were found to be decreased with an IC_{50} of 2.3. Quantitative analysis of total and closed circular HEV RNA by real-time PCR performed on five independent experiments showed that only 1-5% of the HEV RNA internalized at day 1 post-infection entered the core of the cell refinement. The knockdown of 4E-BP1 led to a 1.7 ± 0.6-fold (mean ± SD, n = 5, p<0.01) and 2.4 ± 0.9-fold (mean ± SD, n = 4, p<0.05) (by the clone 56) growth of HEV RNA, respectively. Duncan's multiple range tests were applied to compare the differences between the treatment groups.

Introduction

Hepatitis E virus (HEV) causes acute, enterically transmitted hepatitis in human. It is connected with large epidemics in tropical and subtropical countries where it is endemic or with sporadic cases in non-indigenous countries. Unlike other hepatitis viruses, HEV has several animal reservoirs (Rogée et al., 2013). The virus was first identified in 1983. HEV isolates from humans and other mammals have been broken up in a single species- Hepatitis E virus in genus Hepevirus and family Hepeviridae. HEV is a small (27-34 nm) non-enveloped virus with a single-strand, positive-sense RNA genome of approximately 7.2 kB with three open reading frames (ORFs 1, 2, and 3) (Ahmad et al., 2011).

Higher rates of fulminant hepatitis have been observed in pregnant women (20%) from some endemic regions and in patients suffering from liver conditions caused by hepatitis C virus (HCV) and hepatitis B virus (HBV) infections or by alcohol abuse (Dalton et al., 2011).

Although primary human hepatocytes remain the golden standard, their limited and unpredictable availability, and inability to proliferate, rapid loss of drug metabolizing enzymes and inter individual variability are major disadvantages (Muhammad and Hepa, 2013). HepaRG™ cells exhibit features which make them useful as an *in vitro* model for drug metabolism, disposition and toxicity studies, and could, for many studies and set back the demand for primary human hepatocytes (Andersson et al., 2012).

In the absence of a practical cell culture system, that can facilitate the propagation of HEV *in vitro*, many aspects of the HEV life cycle are even not easily understood. Models of HEV culture *in vitro* have been tackled in primary hepatocytes from non-human primates (Tam et al., 1997). However, none of these culture systems can provide high-titers of infectious HEV in the culture supernatant. In the present experiment, selective toxicity of the nirtetralin was assessed using different molecular and biochemical methods towards the hepatitis E virus cultures.

Materials and Methods

Reagents

Stocks of nirtetralin (Merck, Schiphol-Rijk, Netherlands) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-

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Aldrich, St Louis, MO) with a final concentration of 2 mM. Stocks of LY294022, an inhibitor of PI3K-PKB (Sigma-Aldrich) were dissolved in DMSO. All agents were stored in 15 LL aliquots and frozen at -20°C. Lentiviral particles of GFP-LC3-II (Millipore, Billerica, MA, USA) and expressing GFPLC3 fusion protein were used for visualization of autophagy for-mation. Other reagents including EBSS medium (Lonza), pepstatin A (Santa Cruz Biotech, Santa Cruz, CA) and chloroquine (Sigma-Aldrich) were also used.

HEV cell culture models

HEV genomic RNA was genera-ted from a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) or a construct containing subgeno-mic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-luc), using the Ambion mMESSAGE mMACHINE_ *in vitro* RNA transcription Kit (Life Technologies Corporation) (Shukla et al., 2011; Shukla et al., 2012). The Human hepatoma HepaRG™ cells were collected and centrifuged for 5 min, 1,500 rpm, 4° C. Supernatant was removed and washed with 4 ml optimum by centrifuging for 5 min, 1500 rpm, 4°C. The cell pellet was re-suspended in 100 µL. Optimum and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with the Bio-Rad's electroporation systems using the protocol of a designed program (240 volt, pulse length 0.5, number 1 and cuvette 4 mm) (Shukla et al., 2012). The superna-tant of cultured p6 full-length HEV RNA electroporated cells was collected and used for secondary infection.

Cell culture

HepaRG™ cell lines (human hepatoma cell line) were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies Limited, Hong Kong) complemented with 10% (v/v) fetal calf serum (Sigma Aldrich), 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (Invitrogen-Gibco). Stable firefly luciferase expressing cells were generated by transducing naïve HepaRG™ cells with a lentiviral vector expressing the firefly luciferase gene under control of the human phosphoglycerate kinase (PGK) promoter (LV-PGK-Luc). For visualization of autophagy formation, HepaRG™ cells were transduced with lentiviral vectors expressing the GFP-LC3 fusion protein. Gene knockdown by lentiviral vector delivered short hairpin RNA (shRNA) Lentiviral vectors (Sigma-Aldrich), targeting motor, GFP, were held from the Erasmus Center for Biomics and produced in HEK 293T cells as previously described (Pan et al., 2009). To generate stable gene knockdown cells, HepaRG™ cells were transduced with lentiviral vectors. Since the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 µg/mL puromycin (Sigma) to the cell culture medium. For the infectious model, HEV particles were incubated with knockdown and control HepaRG™ cells.

Measurement of luciferase activity

For Gaussia luciferase, the natural action of secreted luciferase in the cell culture medium was measured using Clarity™ Lumi-nescence Microplate Reader (BioTek Instruments, Winooski, VT).

MTT proliferation assay

10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) (Sigma) was added to cells seeded in 96-well plates and the cells grow at 37°C with 5% CO^2 for 3 hours. The medium was removed and 100 µL of DMSO was added to each well. The visual density of each well was read on the mark Microplate Absorbance Reader (BIO-RAD) at a wavelength of 570 nm. All measurements were performed in triplicates.

Gene expression was examined using quantitative polymerase chain reaction

RNA was isolated with a Viral DNA/RNA Purification Kit (Abnova Corporation, USA) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a Universal RiboClone cDNA Synthesis System (Promega). The cDNA of HEV and GAPDH were amplified by 40 cycles and quantified with a One-Step RT-PCR System (Biorbyt, USA) according to the manufacturer's instructions. GAPDH was considered as reference gene to normalize gene expression. The HEV primer sequences were 5`-ATTGGCCAGAAGTTGGTT TTCAC -3` (sense) and 50-CCGTGGCTATAATTGTG GTCT-3` (antisense), and the primers of housekeeping gene GAPDH were 5`- TGTCCCCACCCCCAATGT ATC-3` (sense) and 5`-CTCCGATGCCTGCTTCACTA CCTT-3` (antisense).

Confocal laser electroscope assay

Lipidated LC3 (LC3-II) is a robust marker of autophagic membranes. Autophagosomes were visualized as bright green fluorescent protein GFP-LC3-II puncta by fluorescence microscopy. For nutrient starvation, cells were incubated in EBSS medium with 1 mM pepstatin A and E-64-d solution overnight prior to fix for confocal laser electroscope analysis. The cells were fixed with 70% ethanol and GFP-LC3-II puncta was detected using confocal electroscope.

Toxicity measurements

Cells were grown to confluence in 96-well flat bottomed tissue culture plates with 3´ 10^4 cells per well and treated with nirtetralin. Concentrations of nirtertralin ranging from 1 to 120 µg/mL were added, each in triplicate cultures in twofold steps. Four days later the

final addition of compound, cultured medium was removed and nirtertralin was added for addition 4 days. MTT solution (10 mL per 100 mL medium) was added to all wells of an assay. Then the plates were incubated at 37°C for 4 hours. Acid isopropanol (100 mL of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to break up the dark blue crystals. After a few minutes at room temperature to insure that all crystals were dissolved, the plates were read with a Micro ELISA reader at a wavelength of 490 nm (Mosmann, 1983; Sargent and Taylor, 1989; Van de Loosdrecht et al., 1994).

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA). Duncan's multiple range tests were used to compare the differences between the treatment groups. A p-value of less than 0.05 was considered to indicate statistical significance. SPSS version 17 was used for all statistical analyses.

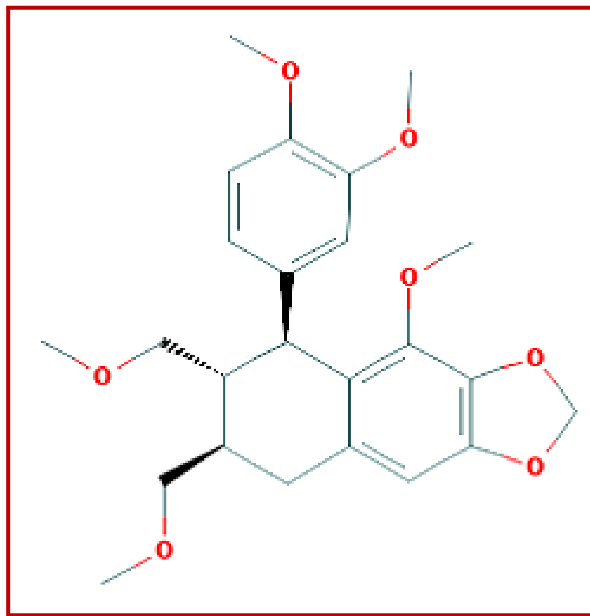

Figure 1: Structure of nirtetralin

Results

The construction of the nirtetralin was found to be similarly previously reported structures (Figure 1). To exclude the possibility that the effects of these compounds on HEV replication represent an mTOR-independent off-target effect, independent confirmation of the role of PI3K/PKB/mTOR signaling cascade in preventing HEV replication was sought through experiments, in which more upstream elements of this signaling cascade were targeted. When HepaRG™, p6-Luc cells were treated with different concentrations of the well-established PI3K inhibitor LY294002, enhancement of HEV replication became apparently similar to that observed with mTOR inhibitors, during the expo-sure of nirtetralin doses. mTOR is a key kinase controlling cellular behavior. Its most important effector pathways include induction of protein transcription via the p70 S6 kinase pathway. However,

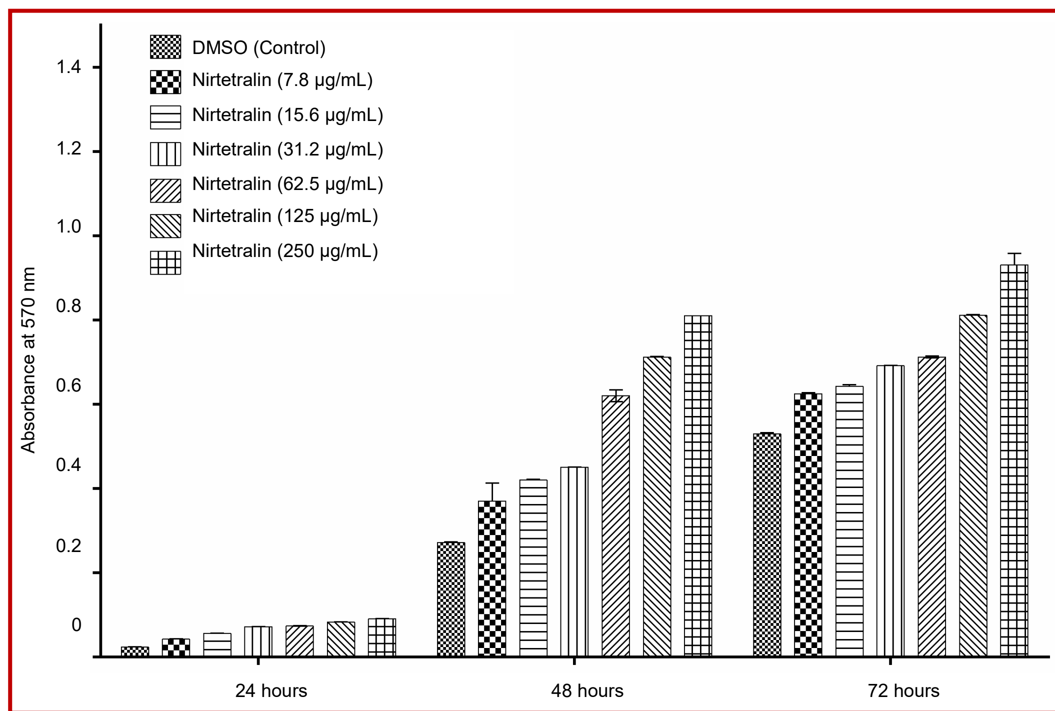

Figure 2: Cell viability of the HepaRG™ cells using the MTT assay

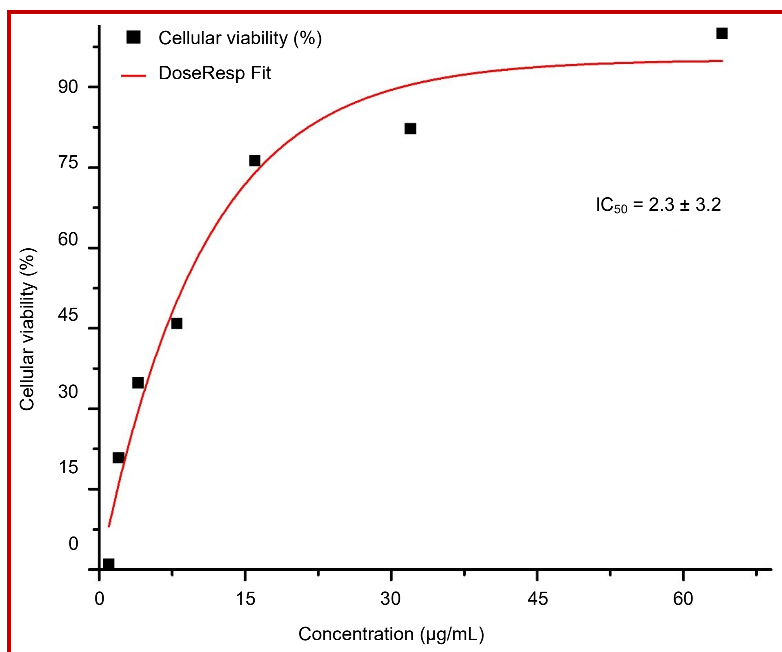

Figure 3: Dose response for the nirtetralin with its IC_{50}

this pathway does not seem to be a major effector mechanism as inhibition of p70 S6 kinase by its inhibitor PF-478671 did not affect HEV infection (Cao et al., 2008).

The HepaRG™ cells transfected with the HEV particles were submitted to several concentrations of nirtetralin, which ensued in a steady and gradual reduction of the viral particles. The viability of the viruses is shown in Figure 2. The IC_{50} was calculated to be 2.3 (Figure 3). The cytopathic effects and plaque formations were registered according to the previous reports. Quantitative analysis of total and closed circular HEV RNA by real-time PCR performed on five independent experiments showed that only 1-5% of the HEV RNA internalized at day 1 post-infection entered the nucleus of the cell culture.

To evaluate the direct effects of nirtetralin, HepaRG™ cells were transduced with integrating lentiviral vectors expressing short hairpin RNA (shRNA) specifically targeting mTOR. The exposure of nirtetralin resulted in the substantial growth of cellular HEV RNA, which was quantified using qRTPCR after inoculation of cell culture produced infectious HEV particles for 72 hours. The knockdown of 4E-BP1 led to a 1.7 ± 0.6-fold (mean ± SD, n = 5, p <0.01) and 2.4 ± 0.9-fold (mean ± SD, n = 4, p<0.05) (by the clone 56) growth of HEV RNA, respectively (Figure 4).

The nirtetralin subjected to the exposure of the HepaRG™ cells with virion particles decreased the expression of the HEV replications, which was confirmed by the green florescence. The luciferase gene expression was involved by the nirtetralin resulting in the lower and decreased expression of the fluorescence (Figure 5).

Discussion

In the present work, it is tempting to speculate that pregnancy-specific down regulation of mTOR may help to understand why this group is specifically sensitive to HEV infection. In seeming agreement, malnutrition in general is too associated with susceptibility to HEV via the nirtetralin exposure among the HepaRG™ cells. Contact exposure with infected animals could lead to an elevated risk of HEV transmission in humans, and recent research by different groups suggests that HEV transmission is possible in relation to the ingestion of raw or undercooked meat and offal from animals (Li et al., 2005). Because of its favorable side-effect profile, nirtetralin (natural based compounds) therapy is quickly gaining popularity for treating a variety of clinical syndromes, especially in oncological disease, in congenital diseases like the Peutz-Jeghers syndrome and the tuberous sclerosis complex, in transplantation medicine and autoimmunity. Therefore, recognition of the anti-HEV function of PI3K-PKB-mTOR pathway bears magnificent implications in clinical practice regarding the choice of particular immunosuppressant for HEV-infected organ transplant recipients. In particular, the use of mTOR inhibitors in these patients should be selected with care. In summation, these solutions may also aid to understand the underlying mechanism why pregnant women are more susceptible to HEV infection with devastating effect. In the present work, the expression HEV RNA in the mobile phone

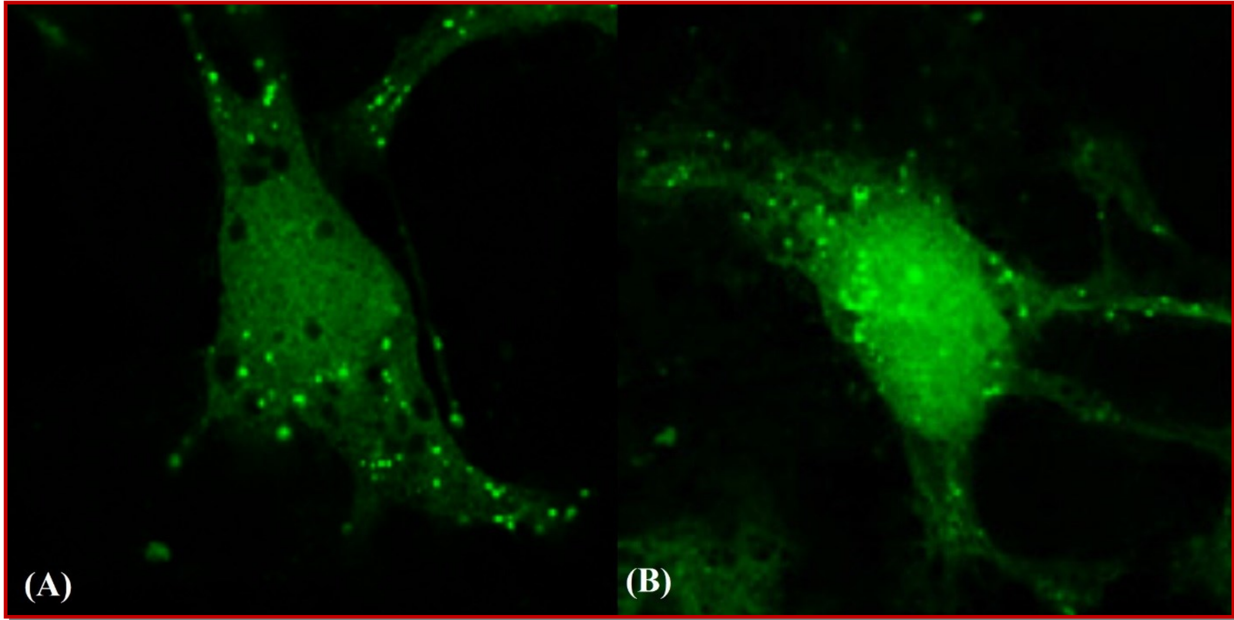

Figure 4: Levels of HBV replication in HepaRG cells are strongly dependent on the viral titre of the inoculum. (A) Control (B) HepaRG cells were infected with inoculum (from 10 to 200 virus genome equivalents per cell) and then cultured in the same conditions with the medium being renewed every 2 days

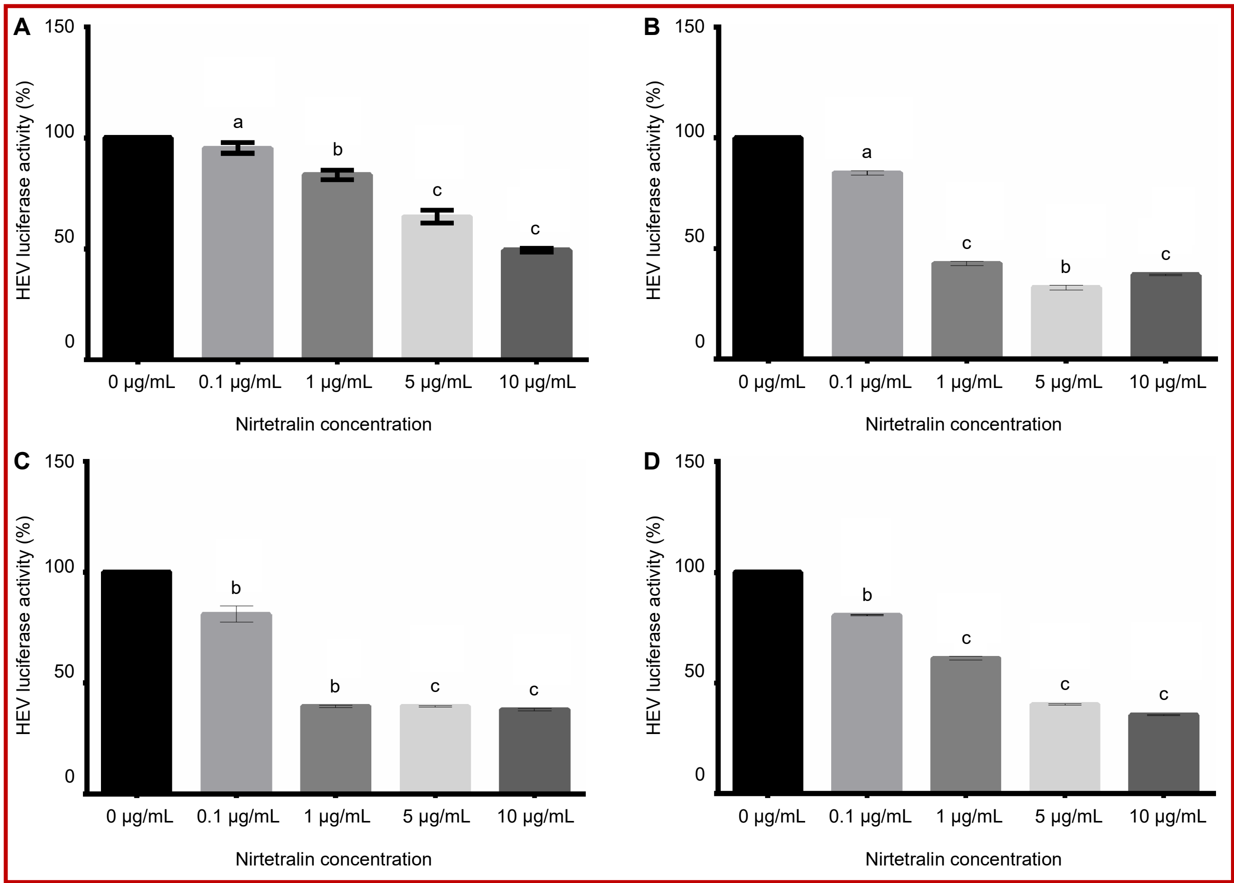

Figure 5: Luciferase gene expression system after (A) 24 hours, (B) 48 hours, (C) 72 hours and (D) Relative HEV RNA expression

culture has resulted in the mimicing of the HEV viral particles in the normal *in vivo* environment and the formation of deceasation of RNA was resulted due to the presence of the nirtetralin.

Conclusion

The use of the nirtetralin or other naturally isolated compounds may pave a new itinerary to the dominance of the viral diseases like hepatitis.

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Conflict of Interest

Authors declare no conflict of interest

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