Int J Pharm Bio Sci 2015 April; 6(2): (P) 141 - 153

Research Article **Pharmaceutics**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

REGULATION OF MATRIXMETALLOPROTEINASE (MMP)-2, MMP-9 AND TISSUE INHIBITOR OF METALLOPROTEINASE (TIMP)-1, TIMP-2 EXPRESSION BY [6]-GINGEROL IN DENGUE VIRUS INFECTED CELL

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ABSTRACT

The role of matrixmetalloproteinase (MMP)-2 and MMP-9 in vascular leakage associated with dengue haemorrhagic fever and MMPs regulation by tissue inhibitor of metalloproteinases (TIMPs) has been established. Control of MMPs and TIMPs activity is of great significance to prevent vascular leakage caused by dengue virus (DV) infection. Gingerol (*Zingiber officinale* Roscoe, Zingiberaceae) is one of the most frequently and heavily consumed dietary condiments throughout the world. This study evaluates the effect of [6] -gingerol on the expression profile of MMP-2, MMP-9 and their tissue inhibitors TIMP-1, TIMP-2 at the MRNA level in DV infected Vero cells. Using quantitative real time RT-PCR, we demonstrated that [6]-gingerol significantly downregulated the mRNA expression of MMP-2 and MMP-9, whereas upregulated the expression of TIMP-1 and TIMP-2 in DV infected cells in a concentration-and time-dependent manner. At high concentrations of [6]-gingerol, TIMP-1 and TIMP-2 were up-regulated after 48 hours of treatment, their over-expression being accompanied by down-regulation of MMP-2 and MMP-9 mRNA expression levels. These results suggest that [6] -gingerol may play a role in regulating vascular leakage by modulating expression of MMP-2, MMP-9 and TIMP-1, TIMP-2 in dengue virus infected cells. Therefore, therapeutic strategies utilizing [6] -gingerol could be developed to substantially reduce dengue morbidity and mortality.

KEYWORDS: Dengue virus (DV), Matrix metalloproteinase (MMP), Tissue inhibitor of metalloproteinase (TIMP), [6] -gingerol

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INTRODUCTION

Dengue fever is an emerging viral disease transmitted by *Aedes aegypti* mosquito arthropods to humans in tropical and subtropical regions of the world, with an estimated occurrence of 50–100 million cases annually [1–4]. According to the World Health Organization, dengue infection is classified as relatively benign fever, called dengue fever (DF), a fatal disease, such as dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) and has resulted in many deaths with an annual estimate of 50 million deaths worldwide [5]. Plasma leakage, as evidenced by hemoconcentration, ascites, or pleural effusion, is the major pathophysiological hallmark that determines disease severity and distinguishes DHF from DF [6]. DV infection induced a number of deregulated circulating factors such as TNFα, MCP1, IL8, IFNγ, VEGF, sVEGFRs and thrombomodulin which may alter endothelial phenotypes leading to dysfunctions in the vascular permeability that may result in severe effusions or even circulatory shock [7-9].The role of matrixmetalloproteinase (MMP)-2 and MMP-9 in vascular leakage associated with dengue haemorrhagic fever and MMPs regulation by tissue inhibitor of metalloproteinases (TIMPs) has been established. MMP with a divalent ZN^{2+} at the active site is an endothelial activating factor that targets components of the extracellular matrix (ECM) and play an important role in immune response to infection by degrading the ECM for leukocyte migration and by modulating cytokine, chemokines and defensin activities [10]. MMPs produced by DV-infected cells have an important role in inducing *in vitro* endothelial cell monolayer permeability. This permeability was associated with a loss of expression of PECAM-1 and vascular endothelium–cadherin cell adhesion molecules and redistribution of F-actin fibres. These in vitro observations have been confirmed in an in vivo vascular-leakage mouse model [11]. It has been shown that DV infection of primary human macrovascular endothelial cells resulted in overproduction of MMP-2 and to a lesser extent, of MMP-9, leading to enhanced endothelial permeability [12]. Moreover, circulating MMP-9, MMP-12 and MMP-13 levels were detected higher in patients during dengue fever compared to healthy individuals. Indeed circulating MMP-9 was associated with disease severity [13]. MMP-2 was significantly elevated in dengue patients with plasma leakage compared to patients without plasma leakage, while MMP-9, TIMP-1 and TIMP-2 was significantly elevated in DV infected patients compared to healthy controls [14]. TIMPs family, TIMP-1, -2, -3 and 4, regulates the activity of multifunctional MMPs. TIMPs inhibit the MMPs activities and could modulate critical signaling pathways independent of metalloproteinase inhibition [15]. As yet, neither vaccine nor specific treatment is available for dengue fever, and patients are currently treated only symptomatically. Consequently, the development of antiviral drugs against dengue viruses remains an urgent need to prevent dengue fatalities. Since the role of MMPs in dengue pathogenesis is well established. It is important to develop the agents that inhibits the MMPs and enhances the TIMPs expression to ameliorate plasma leakage in dengue virus infection. Phytochemicals remain the focus of many *in vitro* studies in the search for compounds against dengue virus [16–18]. Ginger (*Zingiber officinale)* is a natural dietary rhizome that is widely used as a flavoring agent and occasionally used as a traditional medicinal herb. Biologically active compounds of ginger, including [6]-gingerol and shogaols were reported to be effective against *in vitro* model of various disease conditions by modulating the MMP-2 and MMP-9 gene activity, protein expression and secretion [19-22]. However, the efficacy of [6]-gingerol in modulating MMPs and TIMPs cellular response in DHF/DSS has not been explored. This study, therefore, was designed to explore the possibilities if [6]-gingerol could have any such modulating effect on mRNA expression of MMPs and TIMPs in DV infected cells, which might provide the basis for developing novel therapeutic strategies specifically targeting gelatinolytic MMPs and their tissue inhibitors TIMPs and eventually

being useful in controlling endothelial vascular leakage induced in DHF/DSS.

MATERIALS AND METHODS

[6] -gingerol was purchased from Sigma-Aldrich (Singapore) and was dissolved in Dimethyl sulfoxide from Sigma-Aldrich (Singapore) and diluted in cell culture medium. The following reagents and chemicals were obtained from the respective suppliers: Minimum Essential Medium (MEM) and Fetal Bovine Serum (all were purchased from GIBCO Invitrogen Life Technologies, USA). 3-(4, 5-dimethylthiazol-2-yl) -2, 5 diphenyltetrazolium bromide (MTT), was purchased from Sigma-Aldrich, Singapore), Qiagen RNA Isolation Kit was obtained from Qiagen, Germany, Superscript III RT and Real time RT-PCR Master Mix from Bio-Rad Laboratories Inc., Hercules, CA, USA.

(i) Cell Culture and Dengue Virus Propagation

C6/36 cells (Cloned cell line derived from larvae of *A. albopictus*) and Vero cells (*Cercopithecus aethiops* kidney cells) were obtained from the Research and Biotechnology Division (RBD), St. Lukes Medical Center, Quezon City, Philippines. Vero cells and C6/36 cells were grown and maintained in Minimum Essential Medium (MEM, pH 7.2) supplemented with, 20 mM L-Glutamine, 10% heat inactivated fetal bovine serum, 50U/mL penicillin, 50 µg/mL streptomycin (all from GIBCO Invitrogen Life Technologies, USA). Vero cell lines were incubated at 37°C with 5% CO2, whereas C6/36 cell lines were incubated at 28^0C in the absence of CO_2. The clinical isolate of DV3 (strain SLMC-50) was obtained from RBD and was propagated in C6/36 cells, as previously described by Jain *et al.*, with minor modifications [23], and the virus titres expressed as foci-forming units (FFU) were determined by foci formation assay as previously described by Zandi, with slight modifications [24]. For gene expression profiles Vero cells were cultured in serum free MEM before infection with DV and treatment with extracts.

(ii) MTT Cytotoxicity Assay

The MTT assay was carried out according to the method of Denizot and Lang (1986) with slight modifications [25]. Briefly, the trypsinized monolayer cell culture was adjusted to 1 x 10^5 cells/mL using 2% FBS containing MEM. The cell suspension was added to each well of the 96-well microtitre plate. After 24 hrs, the supernatant was aspirated, and the monolayer was washed with the medium and 100 µl of different [6]-gingerol concentrations were added to the cells in microtitre plates. The cells were in an exponential growth phase when the [6] -gingerol were added to the culture. The plates were then incubated for 72 hrs in 5 % CO_2 atmosphere at 37 °C. After 72 hrs, 100 µl of MTT in MEM, 0.5 mg/mL) was added to each well. The plates were gently shaken and incubated for 4 hrs at 37 °C in 5 % CO_2 atmosphere. The absorbance at 570 nm was measured by spectrophotometry (Dynex Technologies Microplate Reader, USA). Non-treated cells were used as negative control (NC). Bleomycin Sulfate was used as positive control (PC). All experiments were done in triplicates. IC_{50} values were generated from the dose-response curves for each cell line. All final concentrations of [6]-gingerol for treatment were adjusted based on the IC_{50}.

(iii) RNA Extraction and Real-time Quantitative RT-PCR for mRNA expression

Vero cells were plated at a density 5X10^4 cell/well with MEM supplemented with 2% FBS and infected with DV at multiplicity of infection (m.o.i) of 1 and incubated in the presence and absence of [6]-gingerol at concentrations of 6.25, 12.5, 25 and 50 µg/ml for 24, 48 and 72 hours of incubation. Non-infected cells (controls) were incubated for all trials. Total RNA was extracted using RNeasy™ mini kit (Qiagen, Germany) according to the manufacturer's protocol. Concentrations and purity of RNA extracts were determined spectrophotometrically by measuring A_{260} and A_{280}. Complementary DNAs (cDNA) were prepared from 1 µg of the total RNA using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions.

Subsequent Real-time PCR amplification was perfomed using SsoFast™ EvaGreen®Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instruction. The PCR was carried out with an initial denaturation at 94°C for 3 min followed by 45 cycles of denaturation at 94°C for 30S, annealing step for 45S (at 56°C for MMP-2 and GAPDH, 59°C for MMP-9, 60^0C for TIMP-1 and TIMP-2) and extension step (at 72°C for 30S), followed by a final elongation (at 72°C for 1 min). GAPDH was used as internal control in each single quantitative RT- Real-time PCR. Real-Time RT PCR was set up and two sets of primers, as depicted in Table 1. Three independent experiments were performed. A real-time quantitative RT-PCR using relative quantitation by the comparative C_T method was used to determine gene expression. Threshold cycle number (C_T), of three independent reactions, was determined using the Rotor Gene RG-3000 Real-Time PCR Detection System and the mean C_T was determined. C_T describes a cycle when the reporter fluorescence dye of a given sample becomes significantly different from the baseline signal. The levels of specific gene expression were normalized to GAPDH levels using the formula $2^{-\Delta\Delta C}T$, where $\Delta\Delta C_T = \Delta C_T$ (sample) – ΔC_T (calibrator) and ΔCT is the C_T of the housekeeping gene (*GAPDH*) subtracted from the C_T of the target genes. The samples are the non-infected Vero cells, [6]-gingerol treated (6.25, 12.5, 25 and 50 µg/ml) treated DV infected Vero cells for 24, 48 and 72 hrs of incubation. Following amplification, melting curve analysis was performed to verify the correct product according to its specific melting temperature (T_m).

Table 1
Primers Sequences

Gene	Forward Primer	Reverse Primer	References
MMP-2	5'-AGGATCATTGGCTACACACC-3'	5'-AGCTGTCATAGGATGTGCCC-3'	[11]
MMP-9	5'-CGCAGACATCGTCATCCAGT-3'	5'-GGATTGGCCTTGGAAGATGA3'	[11]
TIMP-1	5'- GCAACTCCGGACCTTGTCATC- 3'	5'- AGCGTAGGTCTTGGTGAAGC-3'	[26]
TIMP-2	5'- GTAGTGATCAGGGCCAAAG-3'	5'- TTCTCTGTGACCCAGTCCAT-3'	[26]
GAPDH	5'-CCACCCATGGCAAATTCCATGGCA-3'	5'-TCTAGACGGCAGGTCAGGTCCACC-3'	[11]

STATISTICAL ANALYSIS

Statistical methods were adopted using Graphpad prism 6. All the results were expressed as the means ± SD. All experiments were performed three times and data were analysed by ANOVA followed by Tukey's Multiple Comparision test to look for significant differences. P values of < 0.05 were considered statistically significant.

RESULTS

1. Cytotoxicity of [6]-gingerol against Vero cells

Vero cells were treated with [6]-gingerol for 72 hrs. The cytotoxicity effect of [6]-gingerol on Vero cells was determined using MTT assay. The IC50 value of [6]-gingerol was 199.9 µg/mL for Vero cells when added directly to the cells (figure 1). It was determined from this assay that 50 µg/mL [6]-gingerol exerted no significant effects on cell viability and this concentration was used as maximum for all the subsequent studies. There was no observed cytotoxicity for cells treated with 0.1% DMSO, the solvent used to initially dissolve [6]-gingerol.

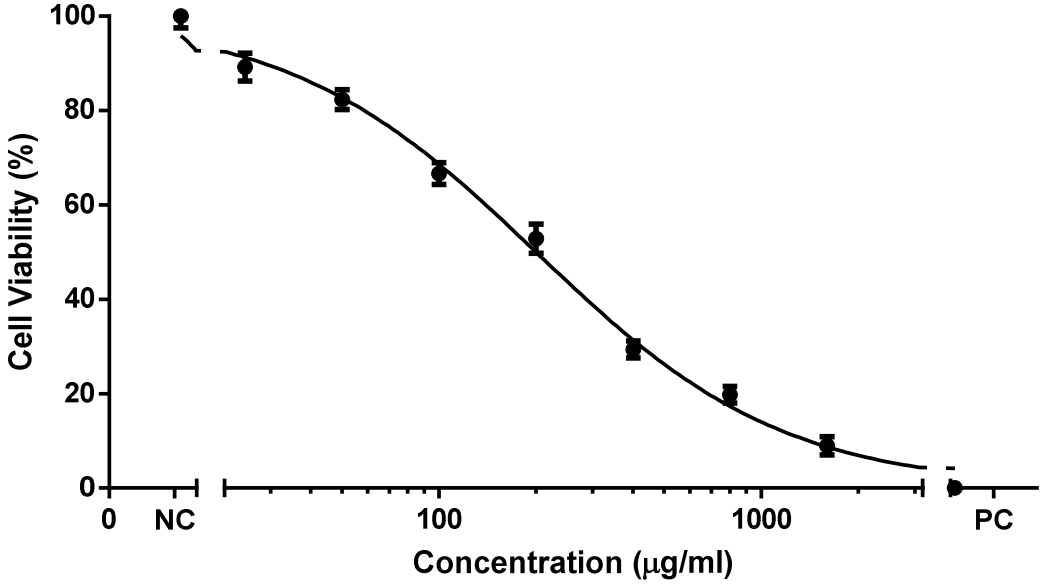

Figure 1
Cytotoxicity of [6]-gingerol on Vero cells. MTT assay was used to evaluate the cytotoxicity of the [6]-gingerol. All experiments were conducted in triplicates.

2. MMP-2, MMP-9, TIMP-1 and TIMP-2 were overexpressed in DV-infected Vero cells

According to the results shown in figure 2, 3, 4 and 5 the mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in Vero cells was significantly influenced by DV-infection. Reverse transcription followed by real-time RT-PCR was employed to analyze the effects of DV-infection on the expression of MMPs and TIMPs. Using real time RT-PCR, we demonstrated that MMP-2 and MMP-9 were overexpressed in Vero cells upon DV infection. MMP-2 was upregulated to 8.74, 9.59 and 9.71 folds ($p<0.0001$) and MMP-9 was upregulated to 5.03, 5.85 and 6.03 folds ($p<0.0001$) in comparison to non-infected cells after 24, 48 and 72 hours of infection respectively. DV infection of Vero cells also upregulated the expression of TIMP-1 to 1.94, 2.06 and 2.70 folds ($p<0.0001$) and TIMP-2 to 2.36, 3.00 and 3.23 folds ($p<0.0001$) in comparison to non-infected cells after 24, 48 and 72 hours of infection respectively, where as that of the internal control (GAPDH) remained unchanged, indicative of enhanced production of natural tissue inhibitors of MMP-9 and MMP-2, respectively.

3. [6]-Gingerol down-regulates MMP-2 mRNA expression in DV-infected Vero cells

Using real-time RT PCR, we demonstrated that [6]-gingerol treatment led to decrease in DV-induced MMP-2 mRNA expression in a dose and time-dependent fashion (figure 2). At the [6]-gingerol concentration of 6.25 µg/mL no difference observed in the MMP-2 gene expression levels in all days compared to the untreated DV infected cell. MMP-2 was reduced significantly to 8.27 folds ($P=0.0021$) and 5.25 folds ($P=0.0011$) in response to [6]-gingerol-treatment with concentrations of 25 µg/ml and 50 µg/ml respectively after 24 hrs of incubation. The expression of MMP-2 was reduced significantly to 9.19 folds ($P=0.0120$) at 12.5 µg/ml, 4.78 folds ($P<0.0001$) at 25 µg/mL and 3.47 folds ($P<0.0001$) at 50 µg/ml in response to [6]-gingerol trearment after 48 hrs of incubation. Similarly the expression of MMP-2 was reduced significantly to 9.22 folds ($P=0.0010$) at 12.5 µg/ml, 3.76 folds ($P<0.0001$) at 25 µg/mL and 2.42 folds ($P<0.0001$) at 50 µg/ml in response to [6]-gingerol trearment after 72 hrs of incubation.

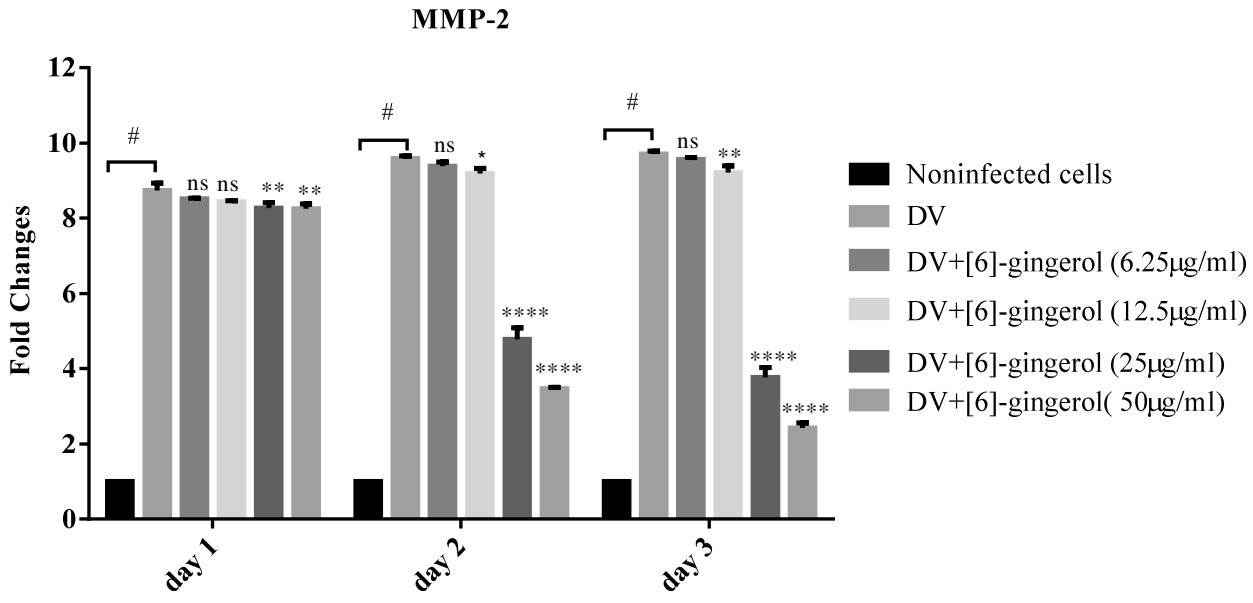

Figure 2

Expression of MMP-2 in non-infected, DV-infected and [6]-gingerol-treated DV-infected Vero cells. For quantitative analysis, total RNA was isolated from non-infected, DV-infected and DV-infected cells after [6]-gingerol-treatment (6.25, 12.5, 25 and 50 µg/ml for 24, 48 and 72 hrs of incubation). RT followed by real time PCR was performed to investigate the gene expression level. Each bar represents the mean ± S.D. calculated from three independent experiments with GAPDH used as the internal control. Columns, mean (n=3); bars, SD. p<0.0001 (#), statistically significant compared with non-infected cells; p = not significant (ns), p<0.1 (*), p<0.01 (**) and p<0.0001 (****), statistically significant compared with DV-infected untreated control.

[6]-gingerol down-regulates MMP-9 mRNA expression in DV-infected Vero cells

The [6]-gingerol exhibited no significant difference in the MMP-9 mRNA expression level at either of treated concentrations for 24 hrs of incubation and at 6.25 and 12.5 µg/ml for 48 hrs of incubation. The expression of MMP-9 was significantly down-regulated in response to [6]-gingerol treatment to 3.74 folds (P<0.0001) and 2.71 folds (P<0.0001) at 25 µg/ml and 50 µg/ml respectively after 48 hrs and to 5.23 folds (P=0.0004), 5.12 folds (P<0.0001), 3.11 folds (P<0.0001) and 2.22 folds (P<0.0001) at 6.25, 12.5, 25 and 50 µg/ml respectively after 72 hrs of incubation (figure 3). These results suggest that [6]-gingerol may induce anti-MMPs effect in time-and-concentration-dependent manner in DV infected cells.

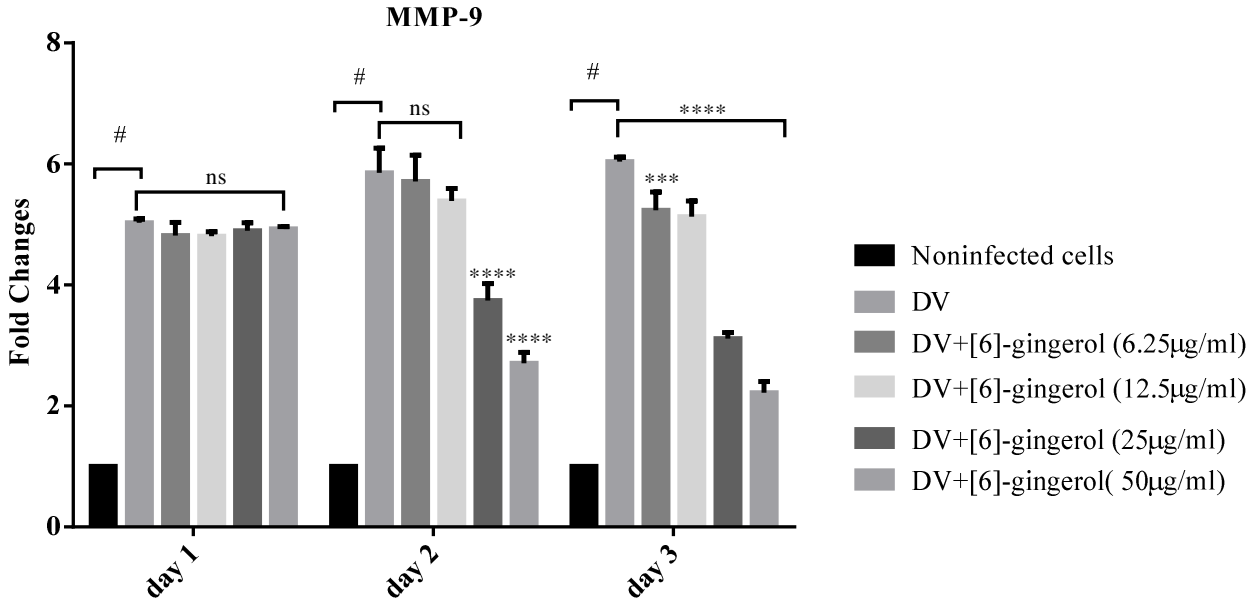

Figure 3

Expression of MMP-9 in non-infected, DV-infected and [6]-gingerol-treated DV-infected Vero cells. For quantitative analysis, total RNA was isolated from non-infected, DV-infected and DV-infected cells after [6]-gingerol-treatment (6.25, 12.5, 25 and 50 µg/ml for 24, 48 and 72 hrs of incubation). RT followed by real time PCR was performed to investigate the gene expression level. Each bar represents the mean ± S.D. calculated from three independent experiments with GAPDH used as the internal control. Columns, mean (n=3); bars, SD. p<0.0001 (#), statistically significant compared with non-infected cells; p = not significant (ns), p<0.001 (***) and p<0.0001 (****), statistically significant compared with DV-infected untreated control.

4. [6]-gingerol up-regulates TIMP-1 mRNA expression in DV-infected Vero cells

After 24 hrs incubation, there was no difference observed in the TIMP-1 mRNA expression at the concentration of 6.25, 12.5, 25 and 50 µg/mL after 24 hrs incubation compared to the untreated DV infected cells. There was no significant difference observed in the TIMP-1 mRNA expression at the concentration of 6.25 µg/mL in all days compared to the untreated DV infected cells. In the [6]-gingerol-treated cells with 12.5, 25 and 50 µg/mL, at 48 hrs of incubation, the expression of TIMP-1 was significantly increased by 2.52 folds (P=0.0001), 2.63 folds (P<0.0001) and 2.92 folds (P<0.0001) respectively than in the untreated DV-infected cells. After 72 hrs incubation, there was a significant increased in the mRNA expression level of the TIMP-1 to 3.25 folds (P=0.0038), 3.25 folds (P<0.0001) and 3.66 folds (P<0.0001) in the [6]-gingerol-treated DV-infected cells with 12.5, 25 and 50 µg/ml respectively (figure 4).

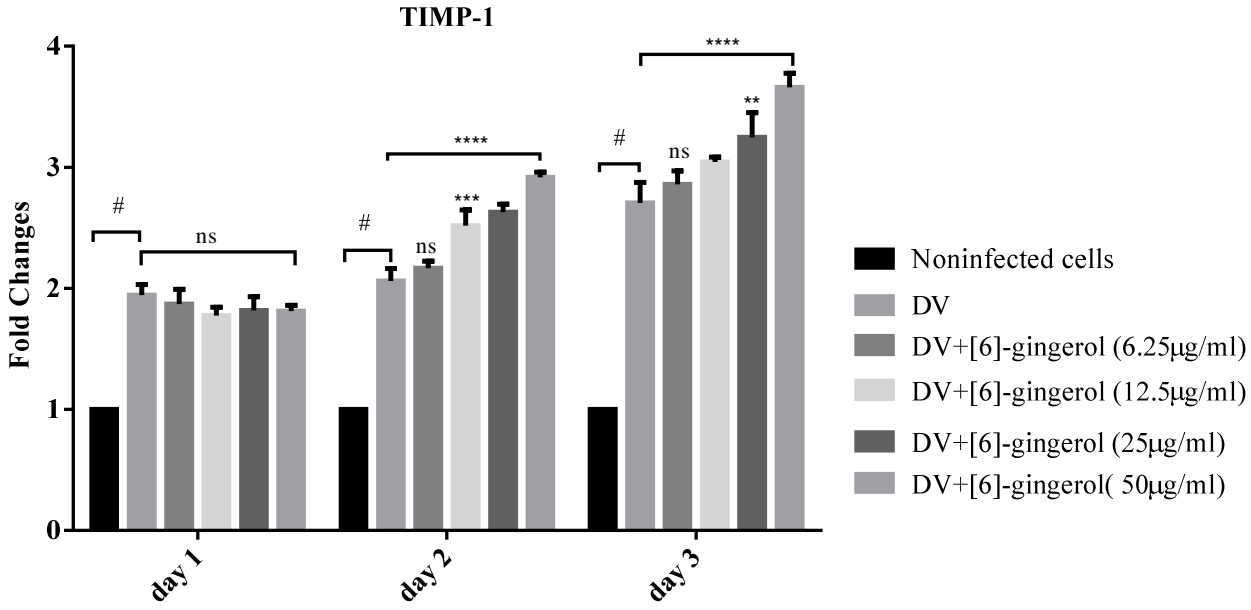

Figure 4

Expression of TIMP-1 in non-infected, DV-infected and [6]-gingerol-treated DV-infected Vero cells. For quantitative analysis, total RNA was isolated from non-infected, DV-infected and DV-infected cells after [6]-gingerol-treatment (6.25, 12.5, 25 and 50 µg/ml for 24, 48 and 72 hrs of incubation). RT followed by real time PCR was performed to investigate the gene expression level. Each bar represents the mean ± S.D. calculated from three independent experiments with GAPDH used as the internal control. Columns, mean (n=3); bars, SD. p<0.0001 (#), statistically significant compared with non-infected cells; p = not significant (ns), p<0.01 (**), p<0.001 (***), and p<0.0001 (****), statistically significant compared with DV-infected untreated control.

5. [6]-gingerol up-regulates TIMP-2 mRNA expression in DV-infected Vero cells

There was no difference observed in the TIMP-2 mRNA expression at the concentration of 6.25 and 12.5 µg/ml at 24 hrs incubation and 6.25 µg/ml at 48 hrs of incubation. The expression of TIMP-2 mRNA was significantly increased in [6]-gingerol-treated DV-infected cells to 2.85 folds (P= 0.0143) and 2.94 folds (P=0.0032) at 25 µg/ml and 50 µg/ml respectively after 24 hrs of incubation and 3.47 folds (P=0.0221), 4.65 folds (P<0.0001) and 6.80 folds (P<0.0001) at 12.5, 25, and 50 µg/ml after 48 hrs of incubation respectively (figure 6). After 72 hrs incubation, there was a significant increased in the mRNA expression level of the TIMP-2 to 3.85 folds (P=0.0016), 4.90 folds (P<0.0001), 6.73 folds (P<0.0001) and 7.04 folds (P<0.0001) in the [6]-gingerol-treated DV-infected cells with 6.25 µg/ml, 12.5 µg/ml, 25 and 50 µg/ml respectively (figure 5). At higher concentrations of [6]-gingerol 25 and 50 µg/ml after 48 hrs of treatment, suggesting that [6]-gingerol induces anti-MMPs effect in a time-and-concentration-dependent manner in DV-infected cells.

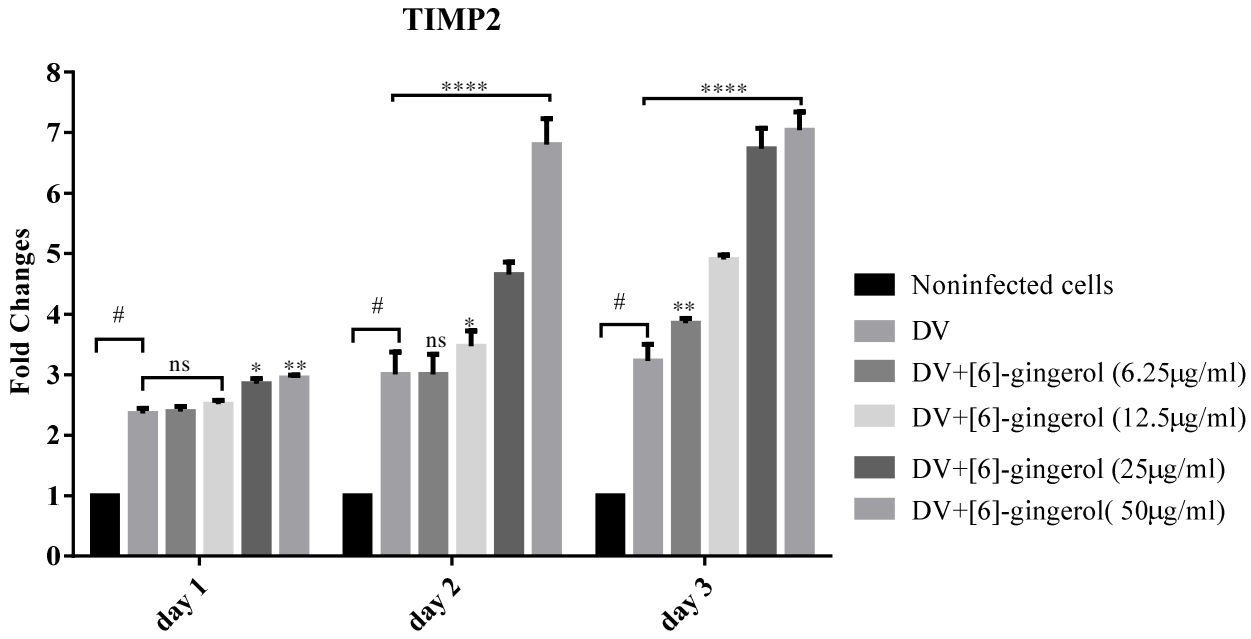

Figure 5

Expression of TIMP-2 in non-infected, DV-infected and [6]-gingerol-treated DV-infected Vero cells. For quantitative analysis, total RNA was isolated from non-infected, DV-infected and DV-infected cells after [6]-gingerol-treatment (6.25, 12.5, 25 and 50 µg/ml for 24, 48 and 72 hrs of incubation). RT followed by real time PCR was performed to investigate the gene expression level. Each bar represents the mean ± S.D. calculated from three independent experiments with GAPDH used as the internal control. Columns, mean (n=3); bars, SD. p<0.0001 (#), statistically significant compared with non-infected cells; p = not significant (ns), p<0.1 (*), p< 0.01 (**), and p<0.0001 (****), statistically significant compared with DV-infected untreated control.

DISCUSSION

A recent study has shown that 390 million persons are infected with DV each year, of which 96 million develop clinical symptoms [27]. A hallmark of dengue disease is an increase in vascular permeability, presenting as pleural effusion and/or ascitis. In severe cases, extensive plasma leakage may lead to the development of hypotension and shock [28]. As yet, neither vaccine nor specific treatment is available for dengue fever, and patients are currently treated only symptomatically. The main strategy to combat dengue infection is through control of the mosquito vector population. However, despite these efforts, dengue infection rates have risen sharply in the last few years. In addition, severe forms of the disease have been occurring with ever increasing frequency, accompanied by rising death rates. On the other hand, vaccine development has been heavily hampered by the complexities of the pathogen itself, the four distinct serotypes, and the likelihood that immune enhancement is playing a role in disease pathogenesis. Consequently, the development of antiviral drugs against dengue viruses remains an urgent need to prevent dengue fatalities. Considering the role of MMPs in vascular leakage during DHF/DSS, it is important to stress that the development of therapeutic approaches for treatment to reduce DV-induced vascular permeability specifically targeting gelatinolytic MMPs and their natural

inhibitors, TIMP-1 and TIMP-2, might be beneficial in controlling vascular leakage-induced in DHF/DSS. MMPs are among the most potent inducers of vascular permeability [29]. The activity of these enzymes is regulated by TIMPs. In this study, we have shown increased expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in DV infected Vero cells compared to non-infected cells which is in agreement with the observations of Luplerdlop *et al*., (2006) and Luplerdlop and Misse, (2008) [11 and 12], who demonstrated that DV-infected cell supernatants had significantly increased level of MMP-2, MMP-9 and their natural tissue inhibitors TIMP-1 and TIMP-2 as compared to non-infected cell supernatants without any restoration of the physiological balance between the MMP and TIMP leading to vascular permeability. Similarly increased levels of circulating MMP-9, MMP-12 and MMP-13 were observed by Kubelka *et al*., (2010) in DF patients and they found higher amount of MMP-9 in patients with the severe form of the illness rather than with the milder variety [13]. Another study conducted by Waidab *et al*., (2008) also demonstrated significant elevation of MMP-9 mRNA expression in children with DHF suggesting the role of this mediator in the pathogenesis [30]. Similarly *Weg et al*., (2014) found significantly elevated levels of MMP-2 in dengue patients with plasma leakage compared to those without any plasma leakage, while MMP-9, TIMP-1 and TIMP-2 were significantly elevated in DV infected patients compared to healthy controls [14]. Interestingly our study showed that the expression of MMP-2 and TIMP-2 were many fold higher as compared to MMP-9 and TIMP-1. Similar observations were noted by Weg *et al*., 2014 [14] who demonstrated a strong correlation between MMP-2 and TIMP-2, suggesting that TIMP-2 could have a central role in modulating MMP-2 activity. In addition, it was also observed that DV infection of microvascular endothelial cells induced secretion of MMP-2 more strongly than secretion of MMP-9 [12]. In contrast, DV infected dendritic cells secreted higher levels of MMP-9 than MMP-2 [11]. The balance between the levels of activated MMPs and free TIMPs determine overall MMP activity. TIMP-1 is a general prototypic inhibitor for most MMP family members and is present in various cell types [31]; however, TIMP-1 has inhibitory activity against MMP-9 [32]. TIMP-2 inhibits proMMP-2 10-fold more effectively than TIMP-1 [33 and 34]. However, TIMP-2 has a bi-functional effect on MMP-2 since MT-MMP mediated proMMP-2 activation requires a tiny amount of TIMP-2 to make activation progress, whereas a greater concentration of TIMP-2 inhibits MMP-2 [35]. Compounds obtained from traditional medicinal plants were shown to possess a variety of active phytochemicals and thus were reported, in the past, to have antiviral and antibacterial activities [22, 36]. *Zingiber officinale* rhizome extracts and its biologically active compounds, including [6]-gingerol and shogaols were reported to be effective against *in vitro* model of various disease conditions by modulating the MMP-2 and MMP-9 gene activity, protein expression and secretion [19-22]. The expression of TIMP-1 was increased in an *in vitro* model of liver cancer following treatment with [6]-gingerol and [6]-shogaols [22]. However, the efficacy of [6]-gingerol in modulating MMPs and TIMPs cellular response in DHF/DSS were not explored. Here, we examined the effects of [6]-gingerol on MMP-2, MMP-9 and TIMP-1, TIMP-2 expression using reverse transcription followed by real-time RT PCR and found that [6]-gingerol significantly downregulated the mRNA expression of MMP-2 and MMP-9, whereas upregulated the expression of TIMP-1 and TIMP-2 in DV infected cells at higher concentrations ([6]-gingerol 25 µg/ml and 50 µg/ml after 48 hrs of treatment), suggesting thereby that [6]-gingerol induced anti MMPs effect in a time-and-concentration-dependent manner in DV-infected cells. In yet another study performed by Weng *et al*., (2010) it was shown that [6]-shogaol and [6]-gingerol exerted anti-invasive activity against hepatoma cells through regulation of MMP-9 and TIMP-1 in a dose dependent manner [22]. Another study by Lee *et al*., (2008) demonstrated that [6]-gingerol inhibited metastasis through dose dependent inhibition of cell adhesion, invasion, motility and activity of MMP-2 and MMP-9 in MDA-MB-231 human breast cancer cells [20]. Similarly Kim

and Kim, (2013) suggested that [6]-gingerol inhibited the invasiveness of pancreatic cancer (PANC-1) cells by decreasing the levels of proteases, MMP-2, and MMP-9 [37]. These reports supported our findings that the natural compound [6]-gingerol played an important anti-MMPs role. Our study is the first of its kind to show that [6]-gingerol treatment could downregulate the mRNA expression of gelatinolytic MMP-2 and MMP-9 while upregulating the mRNA expression of their natural tissue inhibitors TIMP-2 and TIMP-1 in DV infected cells in a way dependent on its concentration and time of interaction. Thus this study identified a new therapeutic approach which may have important role for the treatment of DV-induced vascular leakage by specifically targeting gelatinolytic metalloproteinases. Taking into account the compounds' dietary availability, the ordinary daily human consumption of ginger has approximately 250 mg to 1g and 1.0-3.0% [6]-gingerol and its derivatives [38]. The concentrations of [6]-gingerol [6.25-50 µg/ml) used in this study should be similar to what is present *in vivo*.

CONCLUSION

The results of this study showed that [6]-gingerol modulated MMP-2, MMP-9, TIMP-1 and TIMP-2 genes expression in DV infected Vero cells at transcriptional level in a way dependent on its concentration and time of interaction. To our knowledge, currently there are no data available for the effect of [6]-gingerol on the MMP-2, MMP-9, TIMP-1 and TIMP-2 regarding their expression in DV infected Vero cells in literature. [6]-gingerol may play an important role in regulating DV infection induced vascular leakage by inhibiting the expression of MMP-2 and MMP-9 and increasing the expression of TIMP-1 and TIMP-2. These results provide new insights for the development of anti-dengue agents.

ACKNOWLEDGEMENT

We are thankful to Dr. David Charles Klinzing, Scientist Consultant, St. Lukes Medical Centre for his contribution to the design of the study and supervision of the experiments. This work was supported by the Research and Biotechnology Division (RBD), St. Lukes Medical Centre of Quezon City, Manila, Philippines through grant from the Philippines Council for Health Research and Development (PCHRD) and Department of Science and Technology (DOST) for antiviral research project. We thank Dr. Filipinas Natividad, vice president, RBD for kindly providing the laboratory facility for this study. We also thank to all dengue research groups of RBD for their kind support.

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