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Modulatory activities of *Zingiber officinale* Roscoe methanol extract on the expression and activity of MMPs and TIMPs on dengue virus infected cells

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ABSTRACT

Objective: To evaluate the effect of methanolic extract of *Zingiber officinale* (ZOM) rhizome on the activity and expression profile of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2 at the mRNA level in dengue virus infected Vero cells.

Methods: Total phenolic content and [6]-gingerol content in ZOM were determined by utilizing Folin-Ciocalteu reagent and high performance liquid chromatography. IC_{50} value of ZOM for Vero cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Vero cells were infected with dengue virus to induce MMPs production. Modulatory effect of ZOM on the activity and expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 were demonstrated by using gelatin zymography and real time RT-PCR respectively.

Results: Amount of total phenolics in ZOM in terms of mg gallic acid equivalents/g was (252.89 ± 0.56) and it possessed (137.32 ± 2.47) mg [6]-gingerol content per gram of extract. The IC_{50} value of ZOM was 221.5 μg/mL for Vero cells. The activities of MMP-2 and to a lesser extent MMP-9 were significantly enhanced in the conditioned media collected from the dengue virus infected Vero cells compared to conditioned media from non-infected cells and their activities were significantly inhibited by ZOM in dose-dependent manner. ZOM significantly downregulated the mRNA expression of MMP-2 and MMP-9 and upregulated the mRNA expression of TIMP-1 and TIMP-2 in dengue virus infected Vero cells in concentration-dependent manner.

Conclusions: The results of this study suggest that ZOM may be effective in the control of dengue-virus-induced permeability through the reduction of activities and expression of proteases which degrade the adhesion molecules between cells. This may provide the basis for developing new and effective methods in controlling severe dengue complications.

1. Introduction

Dengue is fast emerging pandemic-prone viral disease in many parts of the world. The World Health Organization (WHO) estimates that 50-100 million dengue infections occur each year and that almost half the world's population lives in countries where dengue is endemic, and currently close to 75% of the global population exposed to dengue are in the Asia-Pacific region[1]. A new classification has been recently developed by WHO for clinicians to make better decisions regarding patient care: dengue, dengue with warning signs, and severe dengue[2]. Severe dengue is characterized by plasma leakage due to altered vascular permeability and slight structural damage to vascular endothelial cells. Dengue fever may progress into the severe form due to abnormalities in hemostasis and increased vascular permeability, eventually leading to dengue shock syndrome via severe plasma leakage[3].

Matrix-degrading metalloproteinases, zinc-dependent endopeptidases, together with inflammatory cytokines *e.g.* interleukin (IL)-1, IL-6, tumor necrosis factor are believed to largely contribute toward the

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progression of severe pathology for dengue infection[4,5]. Matrix metalloproteinases (MMPs) produced by dengue virus infected cells have an important role in inducing *in vitro* endothelial cell monolayer permeability. This permeability is associated with a loss of expression of platelet endothelial adhesion molecule-1 and adhesion molecules of vascular endothelium-cadherin cell and redistribution of F-actin fibres. These *in vitro* observations have been confirmed in an *in vivo* vascular-leakage mouse model[6]. It has previously been found that following dengue virus infection, human macrovascular endothelial cells themselves stimulate the overproduction of MMP-2 and MMP-9 causing the disruption of vascular endothelium-cadherin cell-to-cell adhesion and further enhance endothelial permeability[7]. Moreover, significant elevation of circulating MMP-9 and MMP-2 in dengue patient and their association with the disease severity and plasma leakage compared to healthy controls are well established[8,9].

The role of MMP-2 and MMP-9 in vascular leakage associated with severe dengue and MMPs regulation by tissue inhibitor of metalloproteinases (TIMPs) has been established. TIMPs family, including TIMP-1, 2, 3, and 4, regulates the multifunctional metalloproteinase activities. TIMPs could inhibit the MMP activities and modulate critical signaling pathways independent of metalloproteinase inhibition[10].

Ginger [*Zingiber officinale* (*Z. officinale*)], a member of the Zingiberaceae family, is a popular spice used globally, especially in most of the Asian countries[11]. Ginger and its general compounds such as gingerols, shogaols, paradol and zingerone, possess immunomodulatory, antiapoptotic, anti-tumourigenic, anti-inflammatory, antihyperglycaemic, anti-hyperlipidaemic, antioxidant and anti-emetic activities[12]. Several studies have shown that ginger possesses anti-cancer, anti-microbial, anti-inflammatory, and antioxidant effects[13-18]. [6]-gingerol and shogaols are 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[19-23]. There are neither vaccines nor antiviral drugs to treat dengue fever and severe dengue. Considering the role of MMPs in vascular leakage in dengue virus-infection and modulatory activities of ginger extracts for activity and expression of MMPs and TIMPs in relation to various diseases as demonstrated in previous studies, we evaluated the effect of methanolic extract of *Z. officinale* (ZOM) rhizome on activity and expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in dengue virus-infected Vero cells which may provide the new insight in development of therapeutics to prevent permeability induced by dengue virus-infection.

2. Materials and methods

2.1. Collection and preparation of Z. officinale Rhizome extract

The *Z. officinale* rhizome was collected from the local supplier of Klondykes Tuba, Benguet, Philippines and was authenticated at National Museum, Manila, Philippines. Vouchered specimens were processed at the laboratory of Research and Biotechnology Division (RBD) of St. Luke's Medical Center, Quezon City, Philippines. The fresh ginger rhizomes were cleaned, peeled and cut into cross-sections of (2 ± 1) mm thickness followed by drying at 60 °C and grinding until a fine powder was produced by using a blender.

Extraction protocol followed here was adopted from optimized procedures of the antiviral study group in RBD, St. Lukes Medical Center which was actually adopted from Loh *et al.* in 2009[24]. Briefly, 30 g of powdered rhizome was extracted sequentially with 300 mL each of water, methanol, chloroform and *n*-hexane by using a Soxhlet extractor. The extracts in each solvent for two batches were then combined, filtered and the solvent was removed under reduced pressure by using a rotary evaporator (Heidolph VV2000, Christ®; Alpha 2-4 LSC) at 300 mm Hg at 70 °C. *Z. officinale* aqueous (ZOA) and ZOM extracts were then lyophilized by using a lyophilizer (Lyo Chamber Guard; 121550 PMMA Burket; Germany). The *Z. officinale* chloroform (ZOC) and *Z. officinale* hexane (ZOH) extracts were then dried by using the speed vacuum. The weight of the extracts obtained from the plant material was measured. All extracts were stored at 4 °C until use.

2.2. Total phenolic content

Total phenolic constituents in extracts were determined spectrophotometrically by using the Folin-Ciocalteu reagent, in accordance to the method based on Loh *et al.* utilizing gallic acid as standard[24]. The total phenolic content was determined from the gallic acid calibration curve and was expressed as mg gallic acid equivalents (GAE)/g of extract. All measurements were performed at least in triplicate, and presented as mean ± standard deviation (SD).

2.3. High performance liquid chromatography (HPLC)

Prior to analysis, the standards and samples were filtered through a 0.22 μm syringe filter before injection onto the HPLC system, adopted from Loh *et al.*[24]. The contents of [6]-gingerol in extracts were analyzed by HPLC using Spectra HPLC system (TSP, USA) equipped with P2000 binary pump, AS1000 autosampler, UV2000 UV detector and SW4000 system controller. Twenty μL sample was subjected to HPLC for the [6]-gingerol analysis. Separation was performed on a Lichrospher R_{18} (250.0 mm × 4.6 mm, 5 micron 100A Luna 5u R_{18} column) from Phenomenex USA by maintaining the isocratic binary flow rate (1 mL/min) with a mixture of HPLC grade acetonitrile and water (55:45 v/v). The compounds were identified and quantified based on retention time by using [6]-gingerol as HPLC external standard which was purchased from Sigma–Aldrich, USA (Cat. No. 29150-4). Method was adopted from Puengphian and Sirichote, with minor modifications[25]. A standard curve of [6]-gingerol at the different concentrations of 25, 50, 100, 200, 400 and 800 μg/mL was prepared. The concentration of [6]-gingerol in the extracts was determined by linear regression.

2.4. Virus and cell lines

The clinical isolate of dengue virus serotype 3 (DV-3), strain SLMC-50, was obtained from RBD and was propagated in C6/36 cells, as previously described with slight modification[26]. The virus titres expressed as foci-forming units were determined by focus formation assay as previously described by Zandi, *et al.* with slight modification[27]. C6/36 cells, Vero and Madin-Darby canine kidney cells were obtained from RBD, St. Lukes Medical Center, Philippines. Vero and Madin-Darby canine kidney cells were grown and maintained in minimum essential medium

(MEM) containing 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37 ˚C. C6/36 cells were grown and maintained in MEM containing 10% FBS at 28 ˚C in the absence of CO_2.

2.5. Cytotoxicity assay

The cytotoxicity of ZOM was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described[28]. Confluent Vero cell monolayers were treated in triplicate with increasing concentrations of ZOM. The treated cells were incubated for 3 days at 37 ℃. After 3 days, 100 μL of MTT (Sigma-Aldrich, Singapore) in MEM (0.5 mg/mL) solution was added to each well. The microplate was incubated at 37 ˚C for 4 h in a 5% CO_2 humidified atmosphere. After the incubation period, solubilization/stop solution (100 μL) was added to the wells. The absorbance values of the wells were measured at 570 nm by using a 96-well plate reader (Dynex Technologies Microplate Reader, USA). IC_{50} values were generated from the dose-response curve by using Graphpad Prism 6. All final concentrations of ZOM for treatment were adjusted based on the IC_{50} values. Bleomycin sulfate was used as positive control. Negative control was treated with media alone (without any inhibitors).

2.6. In vitro infection and collection of the conditioned media

Infection of cells with DV-3 and collection of conditioned media were performed according to the method described previously by Luplertlop and Misse with slight modifications[7]. Briefly, monolayer of Vero cells at 2.5×10^5 cells/mL was infected with DV-3 (at a multiplicity of infection = 1) at 37 ˚C for 4 h to allow the virus to adsorp with every 30 min shaking interval, washed twice to remove excess virus, and further cultured at 37 ˚C with 5% CO_2 for 24 h in MEM, without FBS. Uninfected cells (mock infection) were only exposed to dengue-virus-free media and were used as controls. The conditioned cell culture media from dengue virus infected Vero cells was collected in sterile 15 mL conical tube and was concentrated by size-exclusion ultrafiltration by using Amicon TM-4 Centrifugal Filter Units (Millipore). Thus, the collected concentrated conditioned media was aliquoted in 1.5 mL microcentrifuge tubes and stored at -20 ˚C until use. All experiments were performed by using virus-free (negative reverse transcription-PCR) concentrated conditioned media.

2.7. Gelatin zymography

The activity of MMP-2 and MMP-9 in the conditioned medium was determined by gelatin zymography protease assays according to the method described previously[29,30]. Briefly, conditioned media was mixed with nonreducing sample buffer followed by electrophoresis in 8% acrylamide gel containing 1 mg/mL gelatin in the presence of sodium dodecyl sulphate. Following electrophoresis, the gels were soaked in 2.5% Triton X-100 (3 min × 20 min) with gentle agitation to remove the sodium dodecyl sulphate followed by rinsing in distilled water. For gelatinase inhibition assays, ZOM and epigallocatechin-3-gallate (EGCG) were freshly solubilized in the developing buffer used for developing the zymogram; the gel slab was cut into slices corresponding to the lanes which were put in different tanks and incubated in the 10 mL 2X developing buffer, containing 6.67 mL of 1.5 mol/L Tris-HCl buffer (pH 8.8), and 0.4 g $CaCl_2 \cdot {}_2H_2O$ and 0.02% Brij-58 filled to 100 mL with distilled water with or without 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL of the ZOM and 100 μmol/L of EGCG for 20 h at 37 ˚C to allow digestion of the gelatin. After incubation, gelatin activity was visualized by staining gels with 0.1% (w/v) Coomassie blue R-250 in 30% methanol and 10% acetic acid, and destained in the same solution without the Coomassie blue dye. The relative molecular mass of gelatinolytic MMP was determined by comparison with protein molecular weight marker in the adjacent lane. Gel band images were captured by a digital camera and were analyzed for optical density through the Image Lab (Biorad). The assessment of inhibitory activity was based on the measurement of content of clear zone, gelatinolysis of MMP-2 and MMP-9. EGCG was tested for protease inhibitory activity as a control as EGCG caused a strong inhibition of the gelatinolytic activities of MMP-2 and MMP-9[31].

2.8. RNA extraction and real-time quantitative RT-PCR

Gene expression levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 were analyzed with real time RT-PCR as described previously with slight modifications[32]. Briefly, RNA was extracted from non-infected and infected Vero cells with DV-3 (at a multiplicity of infection of 1 foci forming unit/cell) for 48 h at 37 ˚C in presence and absence of various concentrations of ZOM and 100 μmol/L EGCG. After 48 h, RNeasyTM mini kit (Qiagen, Germany) was used according to the manufacturer's protocol. One μg of the total RNA was reverse transcribed by using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Inc., Hercules, CA, USA) and was subjected to quantitative real-time PCR analysis by using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instruction. The relative amount of target mRNA was determined with Ct method by normalizing target mRNA Ct values to those for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ΔCt). The PCR was carried out as denaturation of at 94 ˚C for 3 min followed by 45 cycles, consisted of denaturation at 94 ˚C for 30 seconds, annealing step for 45 seconds (at 56 ˚C for MMP-2 and GAPDH, 59 ˚C for MMP-9, 60 ˚C for TIMP-1 and TIMP-2) and extension step (at 72 ˚C for 30 seconds), followed by a final elongation step (at 72 ˚C for 1 min)[6,33]. The following PCR primers were used for amplifying MMPs and TIMPs. The specificity of the PCR products was confirmed by melt curve analysis. Primers used for amplifying MMPs and TIMPs were as follows: MMP-2 sense: 5'-AGGATCATTGGCTACACACC-3'; antisense: 5'-AGCTGTCATAGGATGTGCCC-3'[6]; MMP-9 sense: 5'-CGCAGACATCGTCATCCAGT-3'; antisense: 5'-GGATTGGCCTTGGAAGATGA-3'[6]; TIMP-1 sense: 5'-GCAACTCCGGACCTTGTCATC-3'; antisense: 5'-AGCGTAGGTCTTGGTGAAGC-3'[33]; TIMP-2 sense: 5'-GTAGTGATCAGGGCCAAAG-3';antisense: 5'-TTCTCTGTGACCCAGTCCAT-3'[34]; GAPDH sense: 5'-CCACCCATGGCAAATTCCATGGCA-3'; antisense: 5'-TCTAGACGGCAGGTCAGGTCCACC-3'[6].

2.9. Statistical analysis

Statistical analysis was performed by using Graphpad prism 6. The

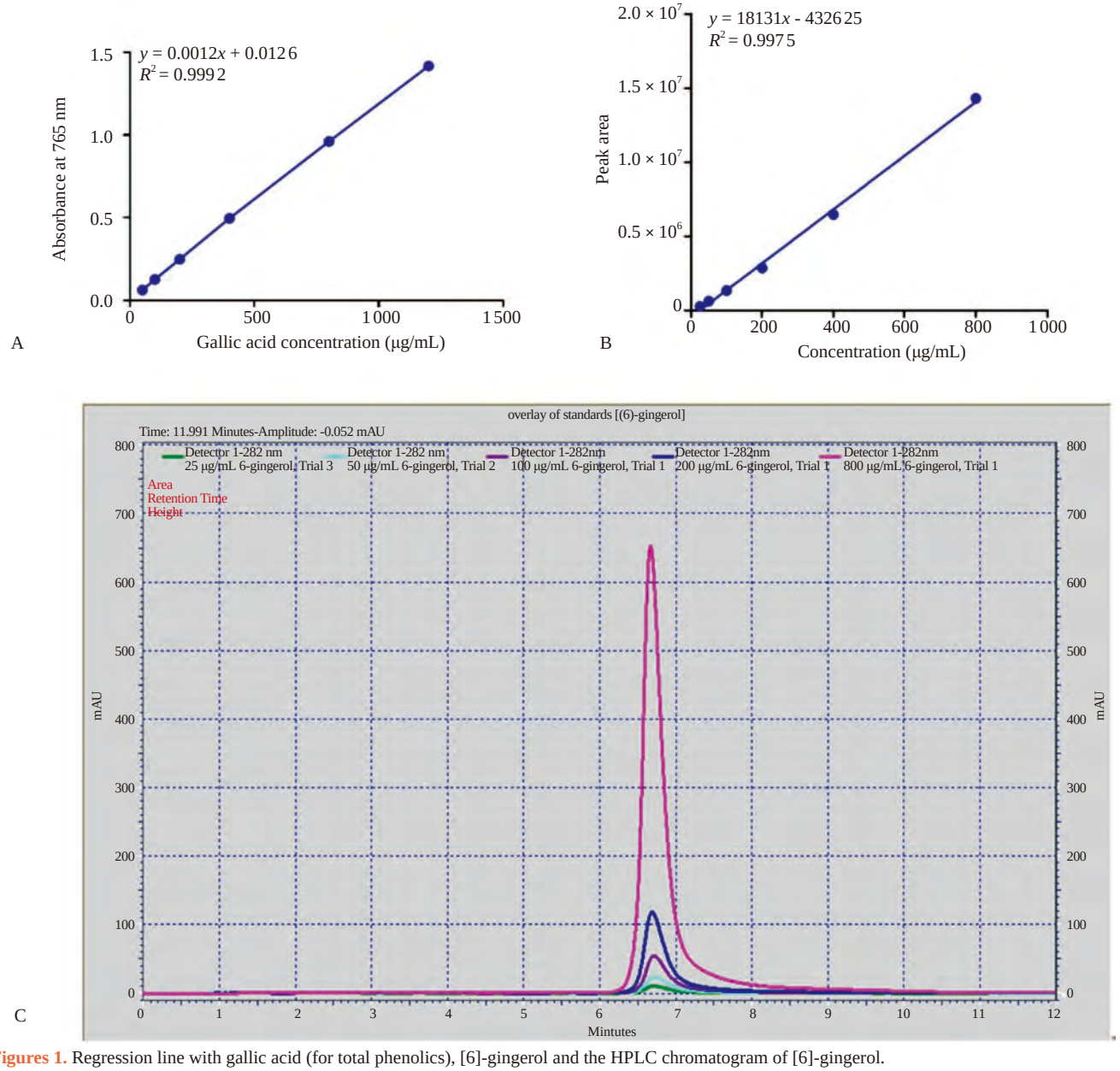
dose-response curve was plotted to determine the IC_{50} values of the extract. All the results were expressed as the mean ± SD. All experiments were performed three times and the statistics were performed by comparing results from treated dengue virus infected cells to untreated dengue virus infected cells (control) by utilizing ANOVA followed by Dunnett's multiple comparision test for significant differences. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Total phenolic and [6]-gingerol content

ZOA, ZOM, ZOC and ZOH had retrieved dry weights of 4.790, 1.706, 2.038 and 0.410 g respectively. In terms of % yield, it has yield of 7.98%, 2.84%, 3.39% and 0.68% respectively. Highly significant quantity of phenolic contents were found in ZOM in terms of equivalents of gallic acid. Equivalents of standards were calculated on the basis of standard regression lines for gallic acid (R^2 = 0.9992) (Figure 1A). Amount of total phenolics in terms of mg GAE/g of dry weight of ZOA, ZOM, ZOC and ZOH were (68.17 ± 0.28), (252.89 ± 0.55), (56.69 ± 0.57) and (47.24 ± 0.58) respectively.

[6]-Gingerol at six different concentrations (25, 50, 100, 200, 400 and 800 μg/mL) was analyzed and peak area responses were noted (average of triplicate injections). Calibration curve was plotted for concentration versus peak area response. The result, by linear regression analysis, showed a very good linear relationship between peak area and concentration. The correlation coefficient was 0.9975. [6]-Gingerol content in ZOA, ZOM and ZOH was calculated using the standard curve (Figure 1B). Among four different extracts, ZOM showed high [6]-gingerol content followed by ZOA and ZOH. [6]-Gingerol was not detected in ZOC. Representative HPLC chromatogram of ZOA, ZOM and ZOH from three independent and triplicate quantifications is shown in



Figures 1. Regression line with gallic acid (for total phenolics), [6]-gingerol and the HPLC chromatogram of [6]-gingerol.
A: Regression line with gallic acid; B: Regression line with [6]-gingerol standards; C: The typical HPLC chromatogram of overlay of [6]-gingerol standards (25, 50, 100, 200, 400 and 800 μg/mL).

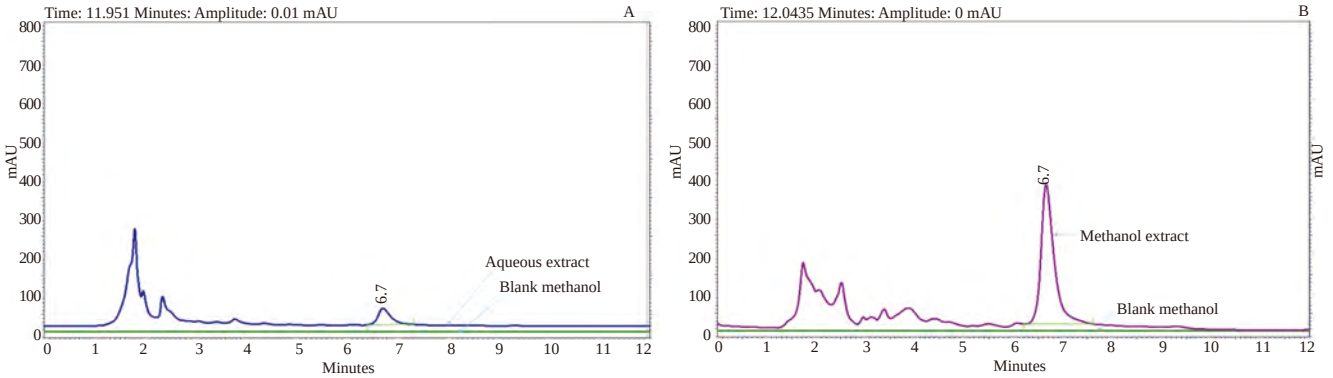


Figure 2. The typical HPLC chromatogram of *Z. officinale* rhizome extracts.
A: ZOA (3 mg/mL) in 55:45 (v/v) acetonitrile-water mobile phase; B: ZOM (3 mg/mL) in 55:45 (v/v) acetonitrile-water mobile phase.

Figure 2A, 2B and 3 and ZOA, ZOM and ZOH possessed (29.32 ± 1.97), (137.32 ± 1.97) and (15.96 ± 1.39) mg [6]-gingerol content per gram of extract respectively. Since ZOM contained significant higher amount of TPC and [6]-gingerol content, it was chosen for subsequent bioassays.

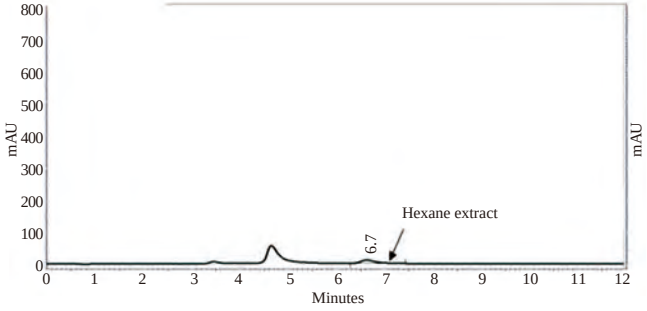


Figure 3. Typical HPLC chromatogram of ZOH (3 mg/mL) in 55:45 (v/v) acetonitrile-water mobile phase.

3.2. Cytotoxic potential of ZOM

The cytotoxic effects of ZOM on Vero cells was determined using MTT assay. The IC_{50} value of ZOM was 221.5 μg/mL (Figure 4). The assay demonstrated that 50 μg/mL ZOM exerted no significant effects on cell viability and this concentration was used as maximum for all the subsequent studies.

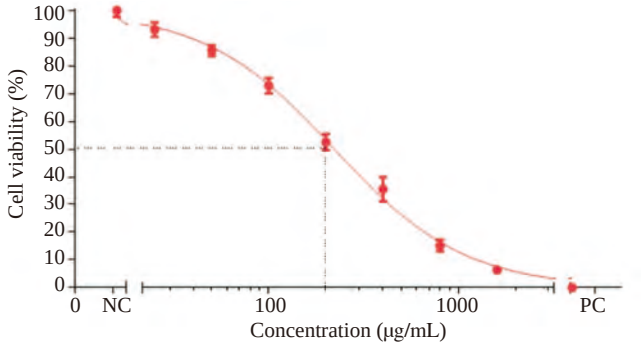


Figure 4. Cytotoxicity of ZOM on Vero cells.
MTT assay was used to evaluate the cytotoxicity of the ZOM. All experiments were conducted in triplicates.

3.3. Inhibitory effects of ZOM on dengue virus induced MMP-2 and MMP-9 activities

The activities of MMP-2 and MMP-9 in conditioned media collected from dengue virus-infected Vero cells was decreased by ZOM treatment in dose-dependent manner (Figure 5). EGCG at 100 μmol/L significantly reduced the dengue virus-induced MMP-2 and MMP-9 activities. MMP-2 activity was reduced significantly to 59.3% ($P < 0.0001$) in response to ZOM-treatment with concentrations of 12.5 μg/mL respectively. Similarly, the activity of MMP-9 was significantly reduced to 84.7.3% ($P = 0.0033$) and 65.0% ($P < 0.0001$) in response to ZOM-treatment with 6.25 and 12.5 μg/mL respectively. There were complete inhibition of MMP-2 and MMP-9 activities at higher concentrations of ZOM (25 and 50 μg/mL). MMP-2 and MMP-9 activities were reduced to 34.3% and 28.5% ($P < 0.0001$) with 100 μmol/L EGCG treatment.

3.4. ZOM transcriptionally regulates MMP-2, MMP-9, TIMP-1 and TIMP-2

The expression of MMP-2, MMP-9 were decreased while the expression of TIMP-2 and TIMP-1 were increased along with an increased concentration of ZOM, whereas that of the internal control (GAPDH) remained unchanged (Figure 6). At the ZOM concentration of 6.25 μg/mL no difference observed in the MMP-2 gene expression levels. MMP-2 was reduced significantly to 0.67, 0.29, and 0.13 folds ($P < 0.0001$) in response to ZOM treatment at concentrations of 12.5, 25 and 50 μg/mL respectively and 100 μmol/L of EGCG (control) significantly reduced the expression of MMP-2 to 0.82 folds ($P = 0.0033$) (Figure 6A). The expression of MMP-9 was significantly down-regulated to 0.81 ($P = 0.0004$), 0.71, 0.47 and 0.39 folds ($P < 0.0001$) in response to ZOM treatment of 6.25, 12.5, 25 and 50 μg/mL respectively. EGCG at 100 μmol/L significantly down-regulated the expression of MMP-9 to 0.78 folds ($P = 0.0001$) (Figure 6B).

There was significant increase in the expression level of the *TIMP-1* gene in the ZOM treated dengue virus infected cells with 6.25, 12.5, 25 and 50 μg/mL to 1.26 ($P = 0.0079$), 2.92, 3.12 and 3.84 folds ($P < 0.0001$) respectively (Figure 6C). Similarly expression of *TIMP-2* gene was significantly increased to 2.58, 4.32, 5.77 and 6.70 folds in ZOM treated cells with 6.25, 12.5, 25 and 50 μg/mL concentration respectively. EGCG significantly upregulated the expression of TIMP-1 to 1.34 folds and TIMP-2 to 2.13 folds in comparison to dengue virus infected cells (Figure 6D). The present study is the first to demonstrate that ZOM significantly downregulated the expression of MMP-2 and MMP-9 and upregulated the expression of TIMP-1 and TIMP-2 in dengue virus infected cells.

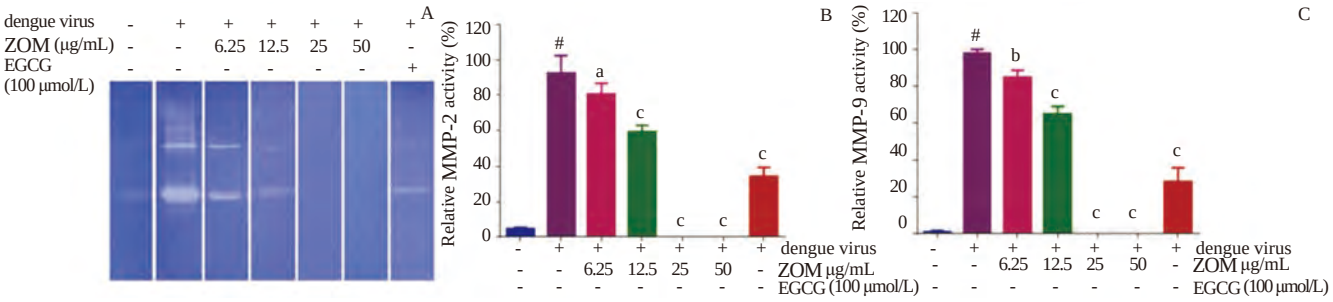


Figure 5. Dose dependent inhibition of MMP-2 and MMP-9 activities after treatment with ZOM.
A: Photograph of the MMP bands, which is representative of three independent experiments; B and C: Quantitative analysis of the bands for MMP-2 and MMP-9 respectively. Each bar represents the mean ± SD calculated from three independent exeriments. Columns represent the mean (n = 3) and bars represent SD. #: P < 0.05 versus control (conditioned media from noninfected cells), a: Not significant; b: P < 0.01; c: P < 0.000 1. The statistics were performed by comparing results from treated conditioned media to untreated dengue virus infected conditioned media control utilizing ANOVA followed by Dunnett's multiple comparision test.

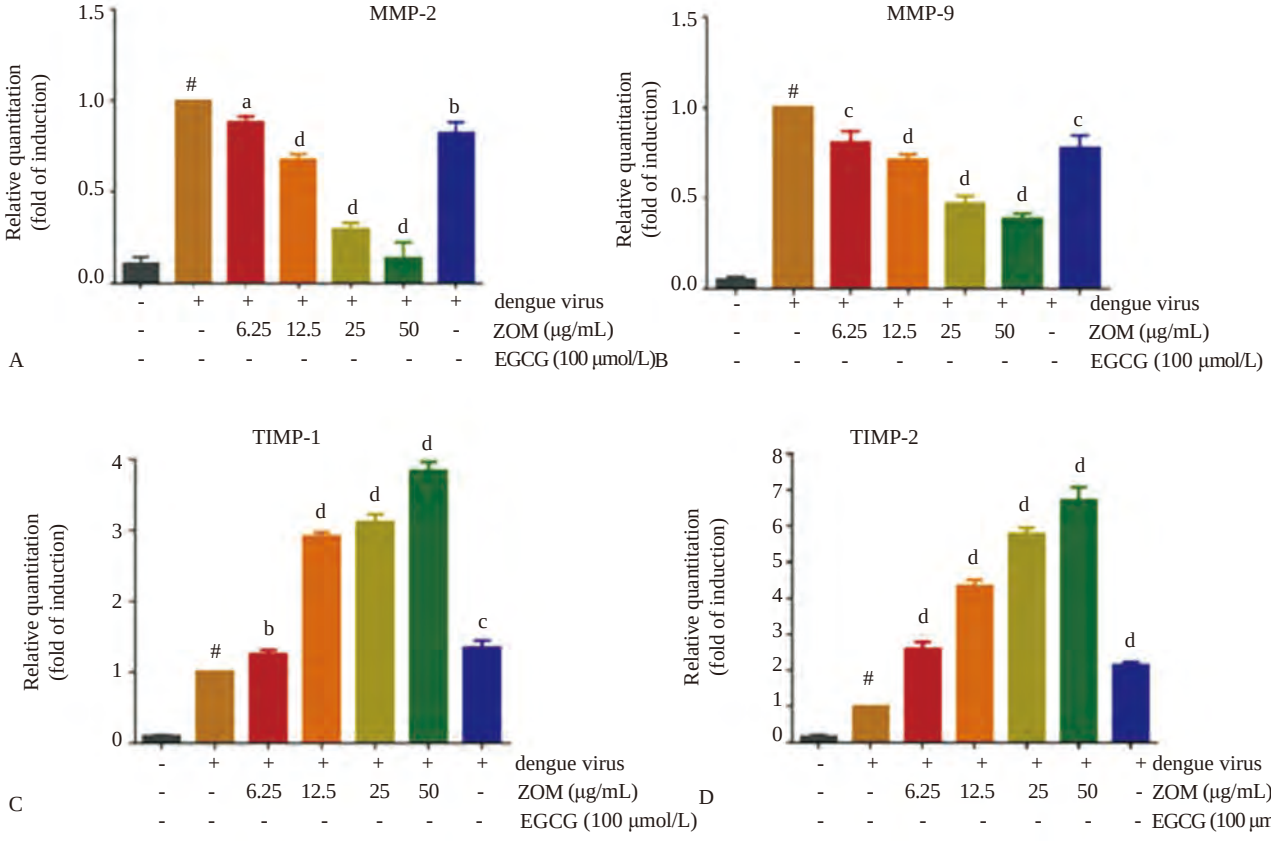


Figure 6. Concentration-dependent inhibitory effects of ZOM on expression of MMPs and TIMPs in dengue virus infected Vero cells.
A: MMP-2; B: MMP-9; C: TIMP-1; D: TIMP-2. Vero cells were plated at a density of 5 × 10^4 cells/mL with MEM supplemented with 2% FBS and infected with DV-3 for 48 h at 37 °C in presence and absence of various concentrations of ZOM and 100 μmol/L EGCG. For quantitative analysis, total RNA was isolated and RT followed by real time PCR was performed to investigate the gene expression level. Each bar represents the mean ± SD calculated from three independent experiments with GAPDH used as the internal control. Columns represent the mean (n = 3) and bars represent the SD. #: P < 0.05 versus control (noninfected cells); a: Not significant; b: P < 0.01; c: P < 0.001; d: P<0.000 1, statistically significant compared with dengue virus-infected untreated control. The statistics were performed by comparing results from treated to untreated dengue virus infected cells utilizing ANOVA followed by Dunnett's multiple comparison test.

4. Discussion

Dengue fever is a major public health problem in several countries of tropical and subtropical zones. An estimation of 50 million infected cases has been reported each year[34]. Disease severities following dengue virus infection are caused by increased vascular permeability leading to hypovolemic shock. For dengue virus infection, MMPs together with inflammatory cytokines *e.g.* IL-1, IL-6, tumor necrosis factor are believed to largely contribute toward the progression of severe pathology[4,5]. A previous study reported that supernatants of dengue virus-infected dendritic cells which contained overproduction of MMP-2 and MMP-9 induced vascular leakage in a mousemodel[6]. Human macrovascular endothelial cells themselves stimulated the overproduction of MMP-2 and MMP-9 causing the disruption of vascular endothelium-cadherin cell-cell adhesion and further enhanced endothelial permeability[7]. Significant elevation of circulating MMP-9 and MMP-2 in dengue patient and their association with the disease severity and plasma leakage compared to healthy controls is well

established in clinical investigation of dengue virus infection[8,9]. In addition, peripheral blood cells from patients who developed dengue henorrhagic fever showed significantly greater expression of MMP-9 than those with milder, dengue fever stage[35].

Despite the various pathogenic mechanisms that have been described for dengue virus infection induced vascular leakage, there are no therapeutics available beyond supportive care and untreated complicated dengue fever can have a 50% mortality rate. Considering the role of MMPs and TIMPs in dengue virus induced vascular leakage, modulation of their activity and expression is of great significance to prevent vascular leakage caused by dengue virus infection. In this study we investigated the modulatory effect of ZOM and established MMPs inhibitor, such as EGCG at 100 μmol/L on the activity and expression profile of MMP-2, MMP-9 and their tissue inhibitors TIMP-1, TIMP-2 at the mRNA level in dengue virus infected Vero cells.

In ZOM, amount of total phenolics in terms of mg GAE/g of dry weight of extracts was (252.89 ± 0.56). [6]-Gingerol is the most abundant pungent compound present among other gingerols in ginger rhizomes, we did quantitation of [6]-gingerol content in the extracts and found that ZOM possessed (137.32±1.97) mg [6]-gingerol content per gram of extract. Results from this study demonstrated the dose dependent inhibition of MMP-2 and MMP-9 activities by ZOM in conditioned media collected from dengue virus-infected Vero cells as demonstrated by gelatin zymography. Similarly, ZOM had strong concentration-dependent modulatory activity on mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. ZOM significantly down-regulated the mRNA expression of MMP-2 and MMP-9, whereas up-regulated the expression of TIMP-1 and TIMP-2 in dengue virus infected cells in dose-dependent manner as demonstrated by real time RT-PCR. The anti-MMPs activity of *Z. officinale* rhizome extract has been attributed to its naturally occurring compounds such as gingerols and shoagols. The increase in the total phenolics content and concentration of [6]-gingerol as the concentration of the extract was increased may account for the concentration-dependent modulatory activities of ZOM on MMPs and TIMPs activities and mRNA expression. In our previous study, we have showed that [6]-gingerol modulated *MMP-2*, *MMP-9*, *TIMP-1* and *TIMP-2* genes expression in dengue virus infected Vero cells at transcriptional level in a way dependent on its concentration and time of interaction[36]. Our observations of modulatory effect of ZOM on MMPs and TIMPs activity and expression are supported by findings of other studies performed earlier, for example, Lee *et al.* 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[21]. Similarly, Weng *et al.* showed 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[23]. Kim and Kim also suggested that [6]-gingerol inhibited the invasiveness of pancreatic cancer cells by decreasing the levels of protease, MMP-2, and MMP-9[37]. This study is of first type showing the modulatory role of *Z. officinale* rhizomes extracts on the activity and expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in dengue virus infected cells.

The role of MMPs in dengue virus-infection induced vascular leakage is well established and the present study demonstrated the modulatory effect of ZOM on activities of MMP-2, MMP-9 and mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in dengue virus infected cells. These findings might provide the new insight for developing therapeutic strategies in controlling endothelial vascular leakage induced in dengue henorrhagic fever/dengue shock syndrome.

Conflict of interest statement

We declare that we have no conflict of interest.

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