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**Original Research Article**

# *Zingiber officinale* Roscoe Aqueous Extract Modulates Matrixmetalloproteinases and Tissue Inhibitors of Metalloproteinases Expressions in Dengue Virus-infected Cells: Implications for Prevention of Vascular Permeability

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## *Abstract*

***Purpose:*** *To investigate the effect of the aqueous extract of Zingiber officinale Roscoe. (ZOA) rhizome on the activity and expression of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 using an in vitro model of Dengue virus (DV) infection.*
***Methods:*** *Z. officinale rhizomes were extracted with water by continuous shaking for 5 days. The total phenolic content in extract was measured by Folin-Ciocalteu method. High performance liquid chromatography (HPLC) was employed to define qualitative and quantitative content of [6]-gingerol in ZOA. The median inhibitory concentration (IC50) value of ZOA for Vero cells was determined by 3-(4,5 dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To induce MMPs production, Vero cells were infected with DV3. The modulatory effect of ZOA on the activity and expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 were assessed using gelatin zymography and quantitative Real-time polymerase chain reaction (RTPCR), respectively.*
***Results:*** *The yield of the ZOA was 7.98%. Total phenolics in ZOA was 68.17 ± 0.28 mg GAE/g of extract and it contained 29.32 ± 1.97 mg 6-gingerol/g of extract.The half-maximal inhibition concentration ($IC_{50}$) of ZOA was 348.8 µg/mL for Vero cells. DV infection of Vero cells significantly elevated the production of soluble gelatinolytic MMP-2 and to a lesser extent, MMP-9, and their activities were significantly inhibited by ZOA in a dose-dependent manner. A significant down-regulation of MMP-2, MMP-9 mRNA expression and up-regulation of TIMP-1, TIMP-2 mRNA expression were observed in DV-infected Vero cells following treatment with ZOA, and it occurred in a dose-dependent manner.*
***Conclusion:*** *The findings of this study suggest that ZOA may ameliorate plasma leakage in dengue virus infection and decrease the chances of severe dengue complications, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) by inhibiting the activities and expression of MMP-2 and MMP-9 while upregulating the expression of TIMP-1 and TIMP-2.*

***Keywords:*** *Zingiber officinale, Dengue virus, Matrix metalloproteinases, Tissue inhibitor, Vascular leakage, 6-Gingerol*

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## INTRODUCTION

Dengue is one of the most serious infectious diseases in the tropical areas of Asia, Africa and

the Americas [1]. Infection by any of the four serotypes of dengue viruses (DV-1, -2, -3 and -4) may result in either a relatively benign fever, called dengue fever (DF), a fatal disease, such as dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) [2]. DV infection causes an estimated 100 million new cases of DF, 500 000 cases of DHF and 25 000 deaths annually [3].

Disease severities following DV infection are caused by increased vascular permeability leading to hypovolemic shock. Matrix metalloproteinases (MMPs) are believed to play a key role in promoting such severities. MMPs are zinc-dependent endopeptidases and comprise a large family of enzymes with different abilities to degrade specific extracellular matrix (ECM) components [4,5]. The role of MMP-2 and MMP-9 produced by DV-infected cells in inducing *in vitro* endothelial cell monolayer permeability and vascular leakage in mice has been established [6,7]. Moreover, significant elevation of circulating MMP-9 and MMP-2 in dengue patients and their association with disease severity and plasma leakage compared to healthy controls is well established [8,9]. The tissue inhibitor of metalloproteinases (TIMP) family, including TIMP-1, TIMP-2, TIMP-3 and TIMP-4, regulates the multifunctional metalloproteinase activities. TIMPs inhibit MMP activities and modulate critical signaling pathways independent of metalloproteinase inhibition [10]. DV infection enhanced the production of TIMP-1 and TIMP-2 but do not restore the physiological balance between the MMP and TIMP leading to vascular permeability [4,5,11].

In Asian countries, ginger (*Zingiber officinale* Roscoe) has been used as a herbal medicine to treat a wide range of disorders such as inflammation, dyspepsia, nausea, vomiting, pain, the common cold and diarrhoea [12]. Phenylpropanoid-derived compounds including 6-gingerol and shogaols are biologically active components present in ginger. They are reported to be effective against *in vitro* model of various disease conditions by modulating the secretion, activity and expression of MMP-2 and MMP-9 [13-17]. However, the efficacy of ginger extracts in modulating MMP and TIMP cellular response in DHF/DSS has not been explored. Currently, there is neither an approved vaccine nor any anti-viral drug available for prevention and treatment of dengue and patients are treated only symptomatically [18,19]. In this study we evaluated the modulatory effect of *Z. officinale* rhizome aqueous extract on activity and expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. The findings of this study may indicate the potential of ginger extract for clinical use in DV infection, which may perhaps decrease the chance of severe dengue complications, DHF and DSS.

## EXPERIMENTAL

### Collection and preparation of aqueous extract

*Z. officinale* rhizomes used in this study were collected from the local supplier of Klondykes Tuba, Benguet, Philippines and were authenticated at National Museum, Manila, Philippines. Voucher specimens were processed at the laboratory of Research and Biotechnology Division of St. Luke's Medical Center, Quezon City, Philippines. The rhizomes were then cleaned, peeled and cut into sections of 2 ± 1 mm thickness followed by drying at 60 $^{o}$C and grinding in a blender until a fine powder was produced. The powder obtained was packed in polyethylene bags and stored in a refrigerator at 4 $^{\circ}$C for further uses. The extraction protocol used was adopted from optimized procedures of the antiviral study group of Research and Biotechnology Division, St. Lukes Medical Center. Thirty grams of powdered rhizome was kept in the flask filled with 350 mL of water and was subjected for continuous shaking for five days. The plant-water suspension was centrifuged at 15000 rpm for 20 min at 4 $^{o}$C. Supernatant was filtered. The filtrate was then collected and evaporated to near dryness using a rotary evaporator (Heidolph WB 2000) at 300 mmHg at 70 $^{o}$C and lyophilized to obtain *Z. officinale* rhizomes aqueous extract (ZOA). Two batches of 30 g powdered samples were extracted and extracts from two batches were pooled together to avoid batch-to-batch variations.

### Determination of total phenolic contents and [6]-gingerol content in ZOA

Weighed amount of lyophilised ZOA was dissolved in water to a concentration of 3 mg/mL. Total phenolic constituents of ZOA were determined by utilizing the Folin–Ciocalteu reagent, in accordance to a method of Loh *et al* with gallic acid as standard [20]. The total phenolic content was determined from a gallic acid calibration curve and expressed as mg of gallic acid equivalent (GAE) per gram of extract. All measurements were performed at least in triplicate, and presented as mean ± SD.

The standards and samples were filtered through a 0.22 µm syringe filter before injecting onto high performance liquid chromatography (HPLC) system, adopted from Loh *et al* [20]. The contents of 6-gingerol in the extracts was analyzed by HPLC using Spectra HPLC system (TSP, USA) equipped with P2000 binary pump, AS1000 autosampler, UV2000 UV detector and SW4000 system controller. ZOA (20 µL) was subjected to HPLC for the 6-gingerol analysis. Separation was performed on a Lichrospher R18 (250 mm x 4.6 mm, 5 micron 100A Luna 5u R18 column) from Phenomenex USA by maintaining the isocratic binary flow rate (1 ml/min) using a mixture of HPLC grade acetonitrile and water (55:45 v/v). The compounds were identified and quantified based on retention times using 6-gingerol as HPLC external standard which was purchased from Sigma–Aldrich (USA) (Cat. No. 29150-4) [21]. A standard curve of the different concentrations of 6-gingerol at 25, 50,100, 200, 400 and 800 µg/mL was prepared. The concentration of 6-gingerol in the ZOA was determined by linear regression.

**Cell culture and dengue virus propagation**

C6/36 cells, BHK-21 and Vero cells were obtained from the Research and Biotechnology Division (RBD), St. Lukes Medical Center, Philippines. Vero 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 $^{o}$C 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 with slight modification [22].

DV3 was titrated by foci forming assay as described previously by Zandi with slight modification [23]. Briefly, a BHK-21 [C-13] cell monolayer was prepared in 96 wells cell culture microplate. After attaining ~80 % confluency, growth medium was removed and the cells were infected by DV-3 (100 µL of serially diluted infected culture fluid) for 2 h to allow virus attachment and penetration into the host cell. Thereafter, the wells were overlaid with 2 % carboxymethylcellulose in Eagle's Minimum Essential Medium (2 % CMC-EMEM) and incubated for 48 h at 37 °C and 5 % $CO_2$.

Following incubation, wells were washed carefully with 1x phosphate buffered saline until residual CMC-EMEM was completely removed. Thereafter, 5 % formaldehyde (JK Baker, USA) was added to fix the cells for 2 h at room temperature (RT) followed by washing (three times) with PBS. Fixed cells were permeabilized using 50 µL of 1 % Nonidet P-40 (Sigma-Aldrich, Singapore) for 1 h. The cells were washed three times with PBS and blocked using 50 µL of BlockAceTM (Wako, Japan) for 1 h. Following blocking of nonspecific sites and another three washing with PBS, 50 µL of 1:10 dilution of dengue virus-specific monoclonal antibody, clone 6B9, (Dengue Research Group, RBD-SLMC) was used to detect the presence of viral antigens. The reaction was allowed to take place for 1 h at RT.

The cells were then washed three times with PBS and incubated with goat anti-rabbit IgG conjugated with horse-radish peroxidase (HRP) at final concentration of 1:100 (Calbiochem, USA) as a secondary antibody for 1 h at RT. Finally, 50 µL of 3,3'-diaminobenzidine (DAB) peroxidase substrate (Biorad, USA) was added to each well to stain the virus foci. Immunostaining of infected cells was monitored for 5 to 10 min. When color development was observed, the reaction was stopped by filling wells with 200 µL of distilled water. Viral foci were viewed, counted and photographed using Olympus CKX41 inverted microscope equipped with DP21 camera and expressed as Foci-Forming-Unit (FFU).

**MTT cytotoxicity assay**

Cytotoxicity of the ZOA was determined using MTT assay as previously described with slight modifications [24]. Briefly, monolayers of Vero cells were seeded in 96-well microplates and treated with different concentrations of ZOA in triplicates. Cells were treated for three days at 37 °C. At the end of the incubation period, 100 µL of MTT (Sigma-Aldrich, Singapore) in MEM (0.5 mg/mL) solution was added to each well. The microplate was kept at 37 °C for 4 h followed by adding solubilization solution/stop mix to each well. The optical density of the wells was measured at 570 nm using 96-well plate reader (Dynex Technologies Microplate Reader, USA). Median Inhibitory Concentration ($IC_{50}$) values were generated from the dose-response curve using Graphpad Prism 6. All final concentrations of ZOA for treatment were adjusted based on the $IC_{50}$. Bleomycin Sulfate was used as positive control. Negative control was treated with media alone (without any inhibitors).

**Infection of Vero cells with DV3 and collection of conditioned media (CM)**

Infection of cells with DV3 and collection of CM was performed according to a method described previously by Luplertlop and Misse with slight

modifications [7]. Monolayer of 2.5 × $10^5$ cells/mL was exposed to DV3 at multiplicity of infection (MOI) of 1 ffu/cell for 4 h to allow the virus to adsorp with gently shaking of flasks every 30 min, washed twice to remove excess virus, and further cultured at 37 $^oC$ with 5 % $CO_2$ for 24 h in MEM, without FBS. The conditioned cell culture media from DV-infected Vero cells were collected in sterile 15-mL conical tubes and was concentrated by size exclusion ultrafiltration using AmiconTM-4 Centrifugal Filter Units (Millipore). Thus obtained conditioned media were aliquoted in 1.5 mL micro-centrifuge tubes and were stored at -20 $^oC$ until use. All experiments were performed using virus-free (negative reverse transcription–PCR [25] concentrated CM.

**Determination of MMP activity by gelatin zymography**

Substrate gel zymography of the activity of MMP-2 and MMP-9 was performed with a Mini-Protein II apparatus (Bio-Rad), according to a method described previously [26,27].

The concentrated CM was re-suspended in 5X sample buffer; 3.72 mL 1 M Tris-HCl pH 6.8, 0.6 mL 1 % bromophenol blue (Sigma-Aldrich, Singapore) in distilled water, 1.68 mL distilled water, 6 mL glycerol (Invitrogen, USA)]. Sample in buffer 15 µL was loaded in alternating well. Four µL of BenchMarkTM Protein Ladder (Invitrogen, USA) was used as a molecular weight marker. Running conditions were as follows: 70 V for the first 30 min and increased to 100 V until the loaded samples were run at least ¾ of the gel. After electrophoresis, the gels were soaked in 2.5 % Triton X-100 (3 × 20 min) in a plastic container at room temperature and placed in a mechanical rotator for gentle agitation followed by rinsing in distilled water. For gelatinase inhibition assays, ZOA and Epigallocatechin-3-gallate (EGCG) were freshly solubilized in the incubation 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 at 37 °C for 20 h in the 10 mL of incubation buffer, containing 6.67 mL of 1.5 M Tris-HCl buffer (pH 8.8), and 0.4 g $CaCl_2$• $2H_2O$ and 0.02 % Brij-58 filled to 100 mL with distilled $H_2O$ without ZOA (control without inhibitor) or with ZOA (6.25, 12.5, 25 and 50 µg/mL) and 100 µM of EGCG. After incubation, the developing buffer was decanted and the slabs were stained for 15-30 min in 0.1 % (w/v) Coomassie blue R-250 in 30 % methanol and 10 % acetic acid, and de-stained in the same solution without the Coomassie blue dye for 10-30 min. After de-staining, a light translucent band over a blue background was detected for gelatinase activity. Gel band images were captured by a digital camera and were analyzed for optical density through the Image Lab (Biorad). Gelatinase proteins that pass across the gel matrix digest the gelatin substrate incorporated in the gel such that MMP-2 and MMP-9 can be detected at 67 kDa and 92 kDa as clear zones against the dark background [28,29]. EGCG was tested for MMPs inhibitory activity as a control since green tea polyphenols caused a strong inhibition of the gelatinolytic activities of MMP-2 and MMP-9, and of the elastinolytic activity of MMP-12 through the compound EGCG [30]. In order to determine the type of gelatinase observed on the zymograms, 10 mM ethylenediaminetetraacetic acid (EDTA) was added to the buffer during the incubation period. All procedures were performed as they were for gels without EDTA in the incubation buffer.

**Real-time RT-PCR for MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA**

The levels of MMP-2, MMP -9, TIMP-1 and TIMP-2 gene expression were analyzed with real time RT-PCR as described previously with slight modifications [31]. RNA was extracted from Vero cells which were seeded at a density of 5 × $10^4$ cells/mL and infected with DV3 (MOI of 1 ffu/cell) for 48 h at 37 °C in presence and absence of various concentrations of ZOA and 100 µM EGCG. After 48 h, the cells were collected by centrifugation (1500 rpm, 5 min, 4 °C) and total RNA was extracted using RNeasyTM mini kit (Qiagen, Germany) according to the manufacturer's protocol. cDNA was prepared from 1 µg of the total RNA using iScriptTM Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's protocol. Subsequent Real-timePCR amplification was perfomed using SsoFastTM EvaGreen®Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instruction. The PCR was run for denaturation at 94 °C for 3 min followed by 45 cycles, consisted of denaturing at 94 °C for 30S, annealing step for 45S (at 56 °C for MMP-2 and GAPDH, 59 °C for MMP-9, 60 $^oC$ for TIMP-1 and TIMP-2) and extension step (at 72 °C for 30S), followed by a final elongation step (at 72 °C for 1 min) [6,32]. The PCR primers were used for amplifying MMPs and TIMPs are shown in Table 1.

**Statistical analysis**

Statistical analysis was carried out using GraphPad Prism 6 software. All the results were

expressed as Mean ± Standard Deviation (SD). The statistics were performed by comparing results from treated DV infected cells with untreated DV infected cells control using Analysis of Variance (ANOVA) followed by Dunnett's Multiple Comparison Test for significant differences. $P < 0.05$ was considered statistically significant.

## RESULTS

### Total phenolic and 6-gingerol contents of ZOA

The dry extract weight of ZOA was 4.790 g, giving a yield of 7.98 %. As assessed by the Folin–Ciocalteu's method, the total phenolic content of the extract was presented as GAE with reference to the gallic acid standard curve (y = 0.0012x + 0.0126, $R^2$ = 0.9992) plotted using the average absorbance values of triplicate sets of data against concentrations of gallic acid in mg/mL. ZOA contained total phenolics of 68.07 ± 0.16 mg GAE/g of the extract.

HPLC chromatogram of ZOA is shown in Figure 1. B and ZOA contained 29.04 ± 2.63 mg 6-gingerol/g extract.

**Table 1:** Primers used for amplifying MMPs and TIMPs

| Gene | Forward primer (5'-3) | Reverse primer (5'-3) | Reference |
|---|---|---|---|
| MMP-2 | AGGATCATTGGCTACACACC | AGCTGTCATAGGATGTGCCC | [6] |
| MMP-9 | CGCAGACATCGTCATCCAGT | GGATTGGCCTTGGAAGATGA | [6] |
| TIMP-1 | GCAACTCCGGACCTTGTCATC | AGCGTAGGTCTTGGTGAAGC | [32] |
| TIMP-2 | GTAGTGATCAGGGCCAAAG | TTCTCTGTGACCCAGTCCAT | [32] |
| GAPDH | CCACCCATGGCAAATTCCATGGCA | TCTAGACGGCAGGTCAGGTCCACC | [6] |

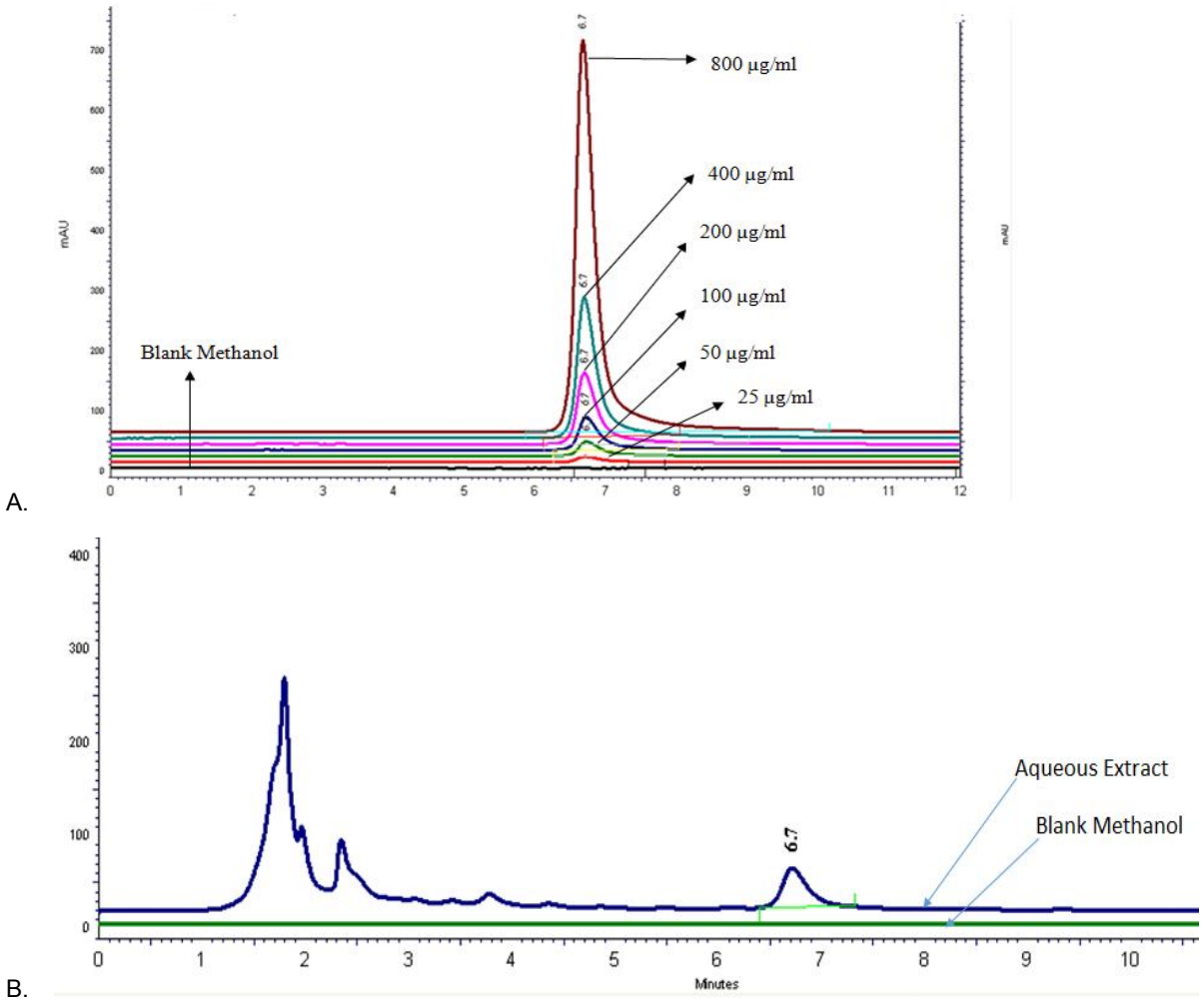


**Figure 1:** 6-Gingerol content of ZOA. A) Typical HPLC chromatogram of A) Overlay of 6-gingerol standards (25, 50, 100, 200, 400 and 800 µg/mL); B) ZOA (3 mg/mL) in 55:45(v/v) acetonitrile-water mobile phase

### Cytotoxicity of ZOA against Vero cells

The $IC_{50}$ of ZOA was 348.8 µg/mL for Vero cells when added directly to the cells (Figure 2). The assay demonstrated that 50 µg/mL ZOA exerted no significant effects on cell viability and this concentration was used as maximum for all the subsequent studies.

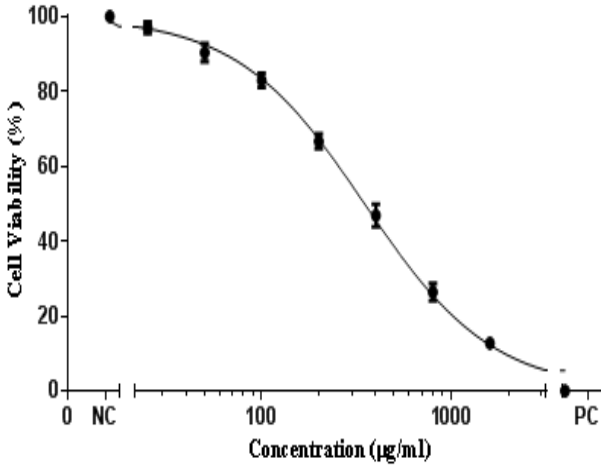


**Figure 2:** Cytotoxicity of ZOA on Vero cells. MTT assay was used to evaluate the cytotoxicity of the ZOA. All experiments were conducted in triplicates

### DV infection of Vero cells triggers overproduction of MMP-2 and MMP-9

DV infection significantly enhanced MMP-2 proteolytic activity at 67 kDa (band corresponded to the molecular weight of MMP-2) and MMP-9 at 92 kDa (band corresponded to the molecular weight of pro-MMP-9) to a lesser extent with $p < 0.0001$ compared to non-infected cells. This enhancement was reduced after treatment with EDTA (metalloproteinase inhibitor, Figure 3).

### Inhibitory effect of ZOA on DV-induced MMP-2 and MMP-9 activities

The activities of MMP-2 and MMP-9 in CM collected from DV-infected Vero cells were decreased by ZOA treatment in dose-dependent manner (Figure 4). 100 µM EGCG significantly reduced the DV-induced MMP-2 and MMP-9 activities. At the ZOA concentration of 6.25 µg/mL treatment, no difference observed in the MMP-2 activity compared to the untreated CM. MMP-2 activity was reduced significantly to 88.7 ($p = 0.0243$), 38.3 and 16.3 % ($p < 0.0001$) in response to ZOA-treatment with concentrations of 12.5, 25 and 50 µg/mL respectively. Similarly, there was no difference observed in MMP-9 activity at CM treated with 6.25 and 12.5 µg/mL of ZOA while the activity of MMP-9 was significantly reduced to 58.3 % and 34.0 % ($p < 0.0001$) in response to ZOA-treatment with 25 and 50 µg/mL respectively. MMP-2 and MMP-9 activities were reduced to 34.3 and 28.5 % ($p < 0.0001$) respectively with 100 µM EGCG treatment.

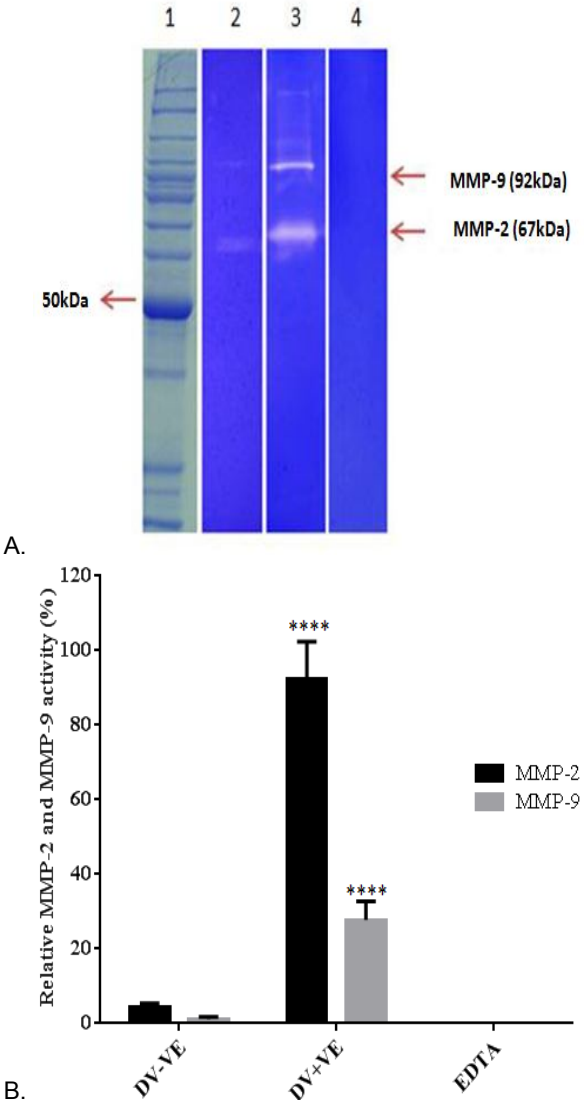


**Figure 3:** Detection of proteolytic activity of MMP-2 and MMP-9 in CM collected from DV infected Vero cells. (A) Using 8 % gelatin-zymography (1-4), showing the ladder (BenchMarkTM, Invitrogen) (1), Concentrated CM from non-infected cells (2), concentrated CM from DV infected cells (3). Concentrated CM from DV infected cells treated with 10 mmol/l EDTA (4). (B) Quantitative analysis of the bands. Each bar represents the mean ± S.D. calculated from three independent experiments; **** above bar indicates $p < 0.0001$ relative to non-infected cells

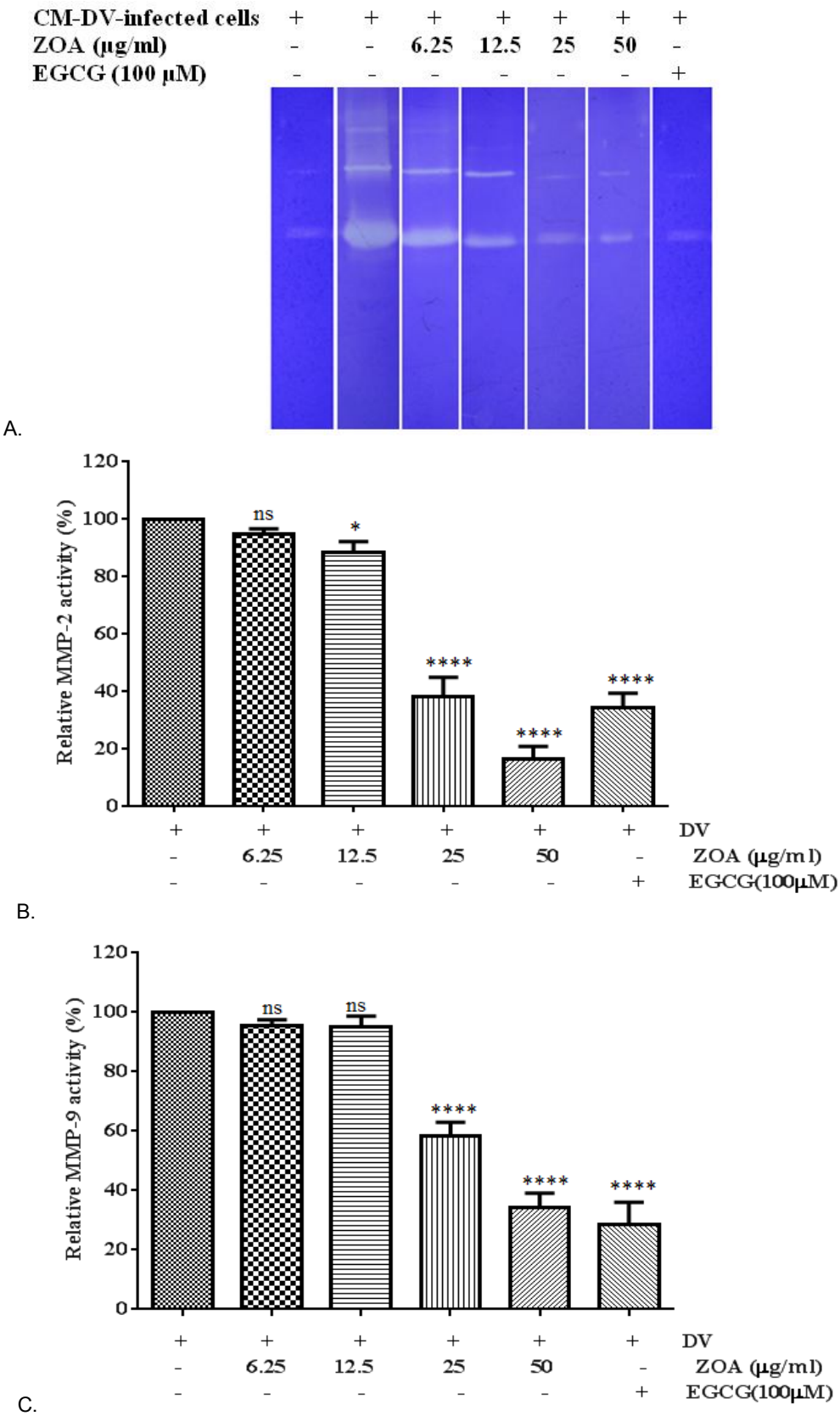


**Figure 4:** Dose dependent inhibition of MMP-2 and MMP-9 activity after treatment with ZOA. (A) Photograph of the MMP bands, which are representative of three independent experiments (A). Quantitative analysis of the bands for MMP-2 and MMP-9 respectively (B and C). Each bar represents the mean ± S.D. calculated from three independent experiments are indicated by not significant (ns), $p < 0.05$ (*) and $p < 0.0001$ (****)

**ZOA transcriptionally regulates MMP-2, MMP-9, TIMP-1 and TIMP-2**

The expression of MMP-2, MMP-9 was decreased while the expression of TIMP-2 and TIMP-1 were increased with an increased concentration of ZOA, whereas that of the internal control (GAPDH) remained unchanged (Figure 5). At the ZOA concentration of 6.25 µg/mL no difference observed in the MMP-2 gene expression levels. MMP-2 was reduced to 0.87 ($p$ =0.0036), 0.56, and 0.19 folds ($p$ < 0.0001) in response to ZOA treatment at concentrations of 12.5 µg/mL 25 µg/mL and 50 µg/mL respectively while 100 µM of EGCG (Control) significantly reduced the expression of MMP-2 to 0.82 folds ($p$ = 0.0002) (Figure 5. A). At 6.25 and 12.5 µg/mL no difference observed in the MMP-9 expression levels. The expression of MMP-9 was significantly down-regulated to 0.82 ($p$ = 0.0001) and 0.76 ($p$ < 0.0001) in response to ZOA treatment of 25 and 50 µg/ml respectively. 100 µM EGCG significantly down-regulated the expression of MMP-9 to 0.78 folds ($p$ < 0.0001) (Figure 5B).

There was significant increase in the expression level of the TIMP-1 gene in the ZOA treated DV infected cells with 12.5, 25 and 50 µg/mL to 1.93, 2.66- and 3.07-fold ($p$ < 0.0001) respectively (Figure 5C). Similarly expression of TIMP-2 gene was increased to 1.92, 3.86 and 4.78 folds in ZOA-treated cells with 12.5, 25 and 50 µg/ml respectively. No difference was observed at 6.25 µg/mL of ZOA treatment. EGCG significantly upregulated the expression of TIMP-1 to 1.34 fold and TIMP-2 to 2.13 folds in comparison to DV infected cells (Figure 5D).

## DISCUSSION

Despite tremendous research efforts for its control and treatment, there is neither vaccine nor specific treatment available for dengue fever, and patients are currently treated only symptomatically. The main strategy for combating dengue infection is through control of the mosquito vector population. On the other hand, vaccine development has been hampered by the complexities of the pathogen itself, the four antigenically distinct serotypes, and the likelihood that immune enhancement is playing a role in disease pathogenesis. Therefore studies of novel therapeutic strategies are needed to improve the clinical outcome of DV infection. The role of MMP-2 and MMP-9 in vascular leakage associated with DHF and MMP regulation by TIMPs has been established [6-9]. Control of MMPs and TIMPs activity is of great significance in preventing vascular leakage caused by dengue virus (DV) infection. Ginger (*Z. officinale* rhizome) is one of the most frequently and heavily consumed dietary condiments throughout the world [33]. 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 [14-17]. However, the efficacy of *Z. officinale* rhizome extracts in modulating MMPs and TIMPs cellular response in DHF/DSS has not been hitherto explored.

In this study, it has been demonstrated that DV infection significantly enhanced MMP-2 proteolytic activity and MMP-9 to a lesser extent. This enhancement was reduced after treatment with EDTA (metalloproteinase inhibitor). These data confirmed that these enzymes are metalloproteinases as these proteinases are dependent upon calcium for their optimal activity, and hydrolyse gelatin.

We investigated the inhibitory effects on the gelatinolytic activity of MMP-2 and MMP-9 by aqueous extract from rhizomes of *Z. officinale*, and by known MMPs inhibitor, such as EGCG. Proteolytic activity was indicated by the presence of clear bands on a dark background and the intensity of the band was shown to be proportional to activity of MMP-2 and MMP-9. Results from this study demonstrate dose-dependent inhibition of MMP-2 and MMP-9 activities by ZOA in conditioned media collected from DV-infected Vero cells as demonstrated by gelatin zymography. Likewise, results presented here confirmed that *Z. officinale* rhizome aqueous extract has strong concentration-dependent modulatory activity on mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. Indeed ZOA significantly downregulated the mRNA expression of MMP-2 and MMP-9, whereas it up regulated the expression of TIMP-1 and TIMP-2 in DV 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. In this study, the amount of total phenolics in ZOA was 68.07 ± 0.16 mg GAE/g of extract and it contained 29.04 ± 2.63 mg 6-gingerol/g of extract. The increase in the total phenolics content and 6-gingerol with increase in concentration of the extract may account for the concentration-dependent modulatory activities of

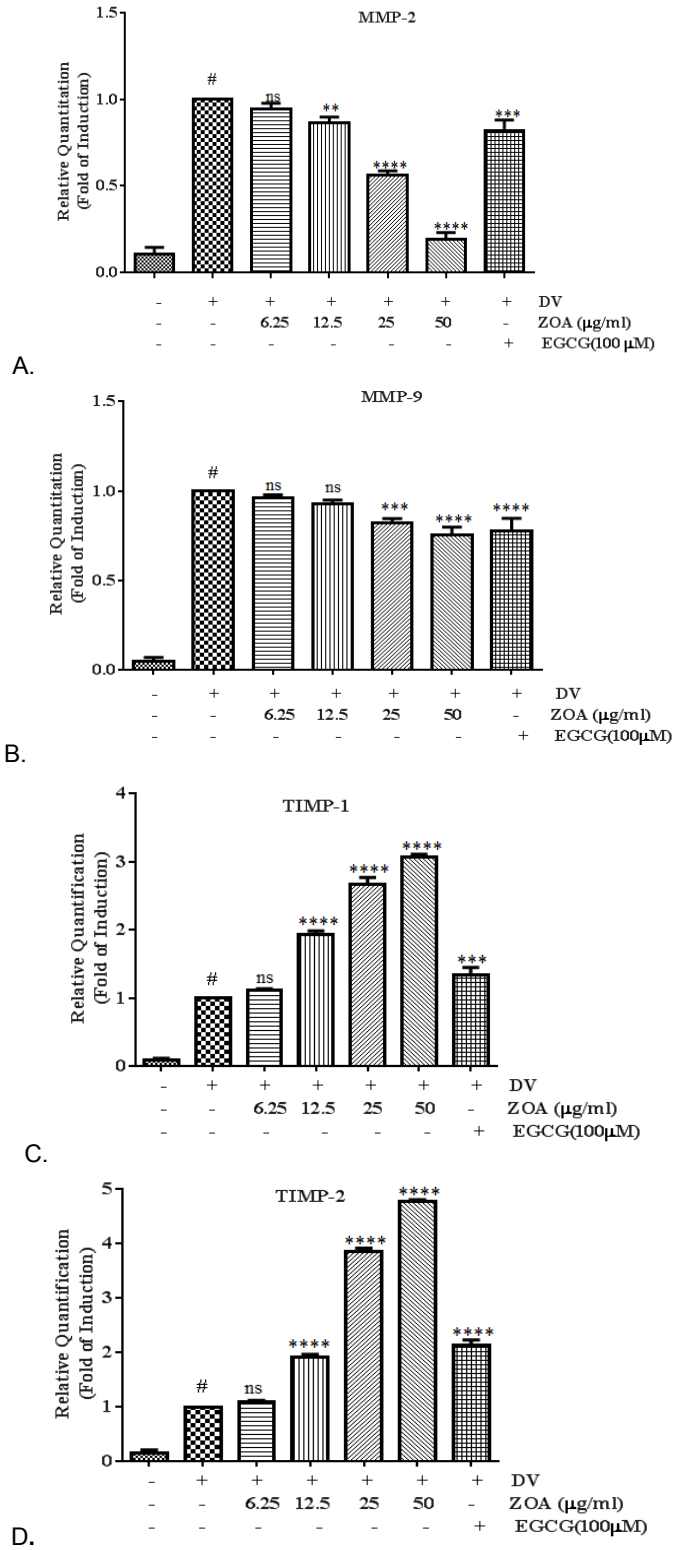


**Figure 5:** Concentration-dependent modulatory effects of ZOA on expression of MMPs and TIMPs in DV infected Vero cells. (A) MMP-2, (B) MMP-9, (C) TIMP-1, (D) TIMP-2. Vero cells were plated at a density of 5x $10^4$ cells/mL with MEM supplemented with 2 % FBS and infected with DV3 for 48 h at 37 °C in presence and absence of various concentrations of ZOA and 100µM 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 ± S.D. calculated from three independent experiments with GAPDH used as the internal control. Columns, mean (n = 3); bars, SD. (#) $p < 0.05$ versus control (noninfected cells), $p$ = not significant (ns), $p < 0.01$ (**), $p < 0.001$(***) and $p < 0.0001$ (****), statistically significant compared with DV-infected untreated control

ZOA on MMPs and TIMPs activities and mRNA expression.

Our observations of modulatory effect of ZOA on MMPs and TIMPs activity and expression are supported by previous findings. 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 [15]. 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 [17]. Kim and Kim also suggested that 6-gingerol inhibited the invasiveness of pancreatic cancer (PANC-1) cells by decreasing the levels of protease, MMP-2, and MMP-9 [34]. All these reports supported our findings that the natural compounds such as 6-gingerol, present in *Z. officinale* rhizomes, play important anti-MMPs role.

## CONCLUSION

The aqueous extract of *Z. officinale* rhizome may play an important role in the regulation of plasma leakage in dengue infection and decrease the chances of severe dengue complications by inhibiting the activity and expression of MMP-2 and MMP-9 while upregulating the expression of TIMP-1 and TIMP-2. Therefore, it can help in the development of new anti-dengue agents.

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