# EFFECT OF *ZINGIBER OFFICINALE* RHIZOME EXTRACTS AND [6]-GINGEROL ON THE ACTIVITY AND EXPRESSION OF MMP-2,-9 AND TIMP-1, 2 INVOLVED IN VASCULAR LEAKAGE IN AN *IN VITRO* MODEL OF DENGUE VIRUS-3 INFECTION

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In Partial Fulfillment
for the Requirements of the Degree
Ph.D. Biological Science

by

BINITA KOIRALA SHARMA

SEPTEMBER 2015

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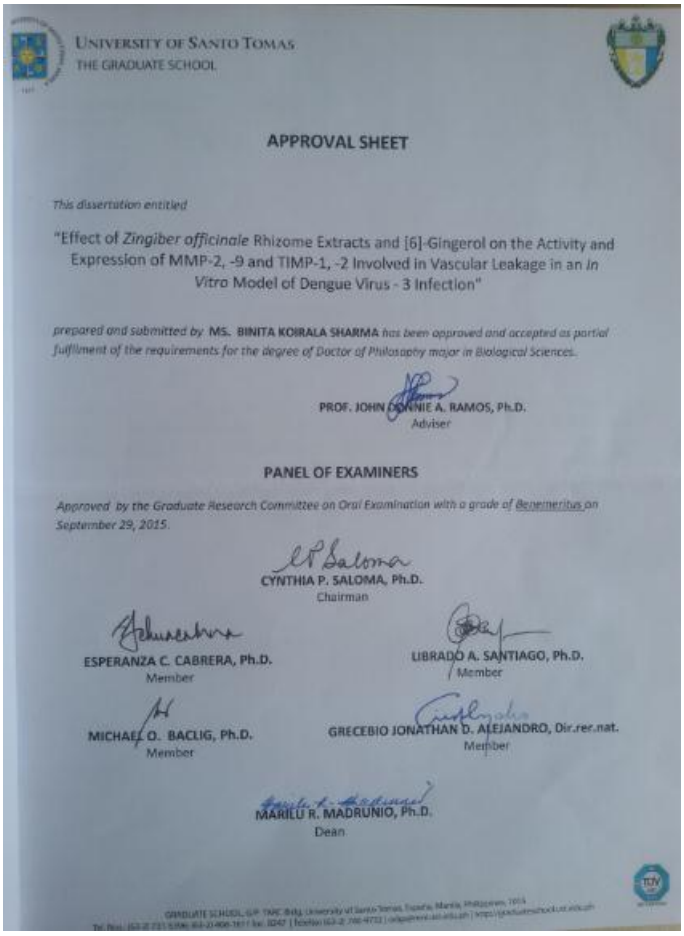

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"Effect of *Zingiber officinale* Rhizome Extracts and [6]-Gingerol on the Activity and Expression of MMP-2, -9 and TIMP-1, -2 Involved in Vascular Leakage in an *In Vitro* Model of Dengue Virus - 3 Infection"

*prepared and submitted by* **MS. BINITA KOIRALA SHARMA** *has been approved and accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy major in Biological Sciences.*

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**ABSTRACT**

Severe dengue following dengue virus (DV) infection is caused by increased vascular permeability leading to hypovolemic shock. The role of matrix metalloproteinases (MMPs) and their regulation by tissue inhibitor of metalloproteinases (TIMPs) have been established in vascular permeability. Control of MMPs and TIMPs activity is of great significance to prevent vascular permeability caused by DV infection. *Zingiber officinale* Roscoe (ZO), is one of the most frequently and heavily consumed dietary condiments with anti-oxidant, anti-inflammatory, immunomodulatory and anti-cancer properties. This research investigated the effects of ZO rhizome extracts and pure [6]-gingerol on the activity and expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 to ameliorate leakage induced by DV infection using an *in vitro* model of DV infection. Total phenolic contents in terms of mg gallic acid equivalents/ gram for methanol (ZOM) and aqueous extract (ZOA) were 252.89 ± 0.56, 68.17±0.28 and possessed 137.32 ± 2.47, 29.32±1.97 mg [6]-gingerol content per gram of extract were determined using high performance liquid chromatography. The median inhibitory concentration ($IC_{50}$) value of ZOM, ZOA were 221.5µg/mL, 348.8µg/mL for Vero cells and 186.4, 368.1µg/mL, respectively for MDCK cells as revealed by the MTT assay. The activity of MMP-2 and to a lesser extent MMP-9 were significantly enhanced in the conditioned media (CM) collected from the dengue virus infected Vero cells compared to CM from non-infected cells ($p<0.0001$). The activities of MMP-2 and MMP-9 were significantly inhibited following treatment with ZOM, [6]-gingerol and ZOA in concentration dependent manner as demonstrated by zymography. mRNA expression of TIMP-1 and TIMP-2 were significantly upregulated accompanied by the downregulation of mRNA expression of MMP-2 and MMP-9 by ZOM, pure [6]-gingerol and ZOA in a dose-dependent manner as demonstrated by qReal time RT-PCR in dose dependent manner. The *in vitro* permeability assay showed that CM from DV-infected Vero cells contain soluble and active factors like MMPs favoring increase of MDCK monolayer permeability compared to CM collected from non-infected cells ($p<0.0001$). ZOM, [6]-gingerol and ZOA reduced the permeability caused by MMPs in a dose dependent manner. The results of this study suggest that *Zingiber officinale* rhizome extracts and pure [6]-gingerol may be effective in the control of DV-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 that warrant further investigation.

*Key words:* Dengue Virus, Matrix metalloproteinase (MMP), Tissue inhibitor of metalloproteinase (TIMP), Vascular leakage, *Zingiber officinale* rhizome

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the Study

Dengue virus (DV serotypes 1–4), a member of the *Flaviviridae* family, has become endemic in most tropical and sub-tropical areas around the world. The World Health Organization (WHO) estimates that 50–100 million dengue infections occur each year and 25,000 deaths annually (WHO, 2002). Almost half the world's population lives in countries where dengue is endemic. Currently, close to 75% of the global population exposed to dengue are in the Asia-Pacific region (WHO, 2012).

In the Philippines, dengue is a major public health concern and a year-round problem, particularly during the rainy season. Outbreaks were reported in 1926 (Siler *et al*., 1926; Simmons *et al*., 1931), and the first recorded epidemic in Southeast Asia occurred in Manila in 1954 (Ooi and Gubler, 2009). Further epidemics occurred in 1966, 1983, and 1998, with increasing numbers of reported cases of dengue disease (Ooi and Gubler, 2009; Gubler, 1997; NOH Philippines, 2005-2010; WHO, 2008). The highest recorded incidence rate (60.9 cases per 100,000 population) and case fatality rate (CFR; 2.6%) has recorded in

the 1998 epidemic. The rising incidence of dengue disease can be explained by several factors including climate change (Russell *et al*., 2009).

Although the mechanism of pathogenesis has not been completely elucidated, it is apparent that increased vascular leakage is the characteristic of dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) (Edelman, 2005; Welsh and Rothman, 2003). The vascular endothelium is a continuous sheet of cells that lines the blood vessels and provides the major blood-tissue barrier. Cells of the vascular endothelium are held together by tight junctions that regulate the permeability of macromolecules. It has been proposed that vascular leakage may be due to disruptions of cell-cell junctions during dengue infection (Basu & Chaturvedi, 2008). The precise mechanisms leading to these conditions are not known but several factors have been hypothesized to be involved including antibody-dependent enhancement (ADE), memory T-cell mediated pathogenesis during a heterologous secondary infection and over expression of proinflammatory cytokines (Basu and Chaturvedi, 2008).

Zinc-dependent endopeptidases, such as matrix metalloproteinases (MMPs), together with inflammatory cytokines *e.g.*IL-1, IL-6, TNFα are believed to largely contribute toward the progression of severe pathology for dengue infection (Martina, 2009; Nielsen, 2009). MMPs are a family of gelatinolytic proteases originally described to degrade the extracellular matrix (ECM). MMPs produced by dengue virus-infected cells have an important role in inducing *in*

*vitro* endothelial cell monolayer permeability (Luplerdlop and Misse, 2008). Cell culture supernatants collected from DV-infected DCs disrupted the platelet endothelial cell adhesion molecule-1 (PECAM-1) and VE-cadherin cell adhesion molecules expression leading to increased vascular permeability in a mouse model (Luplertlop *et al*., 2006). Dengue infected primary human endothelial cells resulted in overproduction of MMP-2 and to a lesser extent of MMP-9, leading to enhanced endothelial permeability *in vitro*, which was associated with loss of expression of the vascular endothelium-cadherin cell-cell adhesion and may contribute to the pathogenesis of severe dengue infection (Luplertlop and Misse, 2008). Moreover, significant elevation of circulating MMP-9 and MMP-2 in dengue patients and association of the level of MMP with disease severity and plasma leakage compared to healthy controls is well established (Kubelka *et al*., 2010; Weg *et al*., 2014).

In addition to the role of MMPs, the regulation of MMP activity through the action of tissue inhibitor of metalloproteinases (TIMPs) has also been established. The TIMPs family of proteins, including TIMP-1, 2, 3, and 4, regulate the multifunctional metalloproteinase activities of MMPs. TIMPs inhibition of MMP activity can also modulate critical signaling pathways independent of metalloproteinase inhibition (Sun, 2010). DV infection has been shown to lead to enhanced production of TIMP-1 and TIMP-2 but is hypothesized to not be able to restore the physiological balance between the MMPs and TIMPs leading to

microvascular leakage (Luplerdlop *et al*., 2006). These data strongly suggest that MMPs and TIMPs could be significant targets in controlling the vascular permeability seen in dengue infection and there is a need to identify a new therapeutic approach for the treatment of viral-induced vascular leakage by specifically targeting metalloproteinase.

No effective vaccine against dengue virus infection is currently licensed, although there are candidate vaccines being tested in phase I and II clinical trials (Webster *et al*., 2009). Furthermore, there are no antiviral drugs to treat dengue fever (DF), DHF, or DSS. Consequently, the development of antiviral drugs against dengue viruses remains an urgent need to prevent dengue fatalities. Compounds obtained from traditional medicinal plants and herbs species have been reported to have antiviral activity and a wide variety of active phytochemicals have been identified (Muliawan *et al*., 2006; Jain *et al*., 2008; and Schnitzler *et al*., 2007).

Ginger (*Zingiber officinale*), a member of the Zingiberaceae family, is a popular spice used globally especially in most of the Asian countries (Demin, 2010). Ginger and its general compounds such as gingerols, shogaols, paradols and zingerone exert immuno-modulatory, anti-apoptotic, anti-tumourigenic, anti-inflammatory, anti-hyperglycaemic, anti-hyperlipidaemic, antioxidant and anti-emetic activities (Rasmussen, 2011). Several studies have shown that ginger possesses anti-cancer, antimicrobial, anti-inflammatory, and antioxidant effect

(Liu *et al*., 2012; Bellik *et al*., 2014; Rasvidah *et al*., 2014; Hu *et al*., 2012; Lee *et al*., 2012; Rahman *et al*., 2011). [6]-gingerol and shogaols are reported to be effective in *in vitro* models of various disease conditions by modulating the MMP-2 and MMP-9 gene expression, protein expression, secretion and activities, (Kundu *et al*., 2009; Yanti, 2011; Lee *et al*., 2008; Ling *et al*., 2010; Weng *et al*., 2010).

Hitherto, no studies have been accomplished to assess the possible protective effects of *Z. officinale* rhizome extracts and its primary component, [6]-gingerol, against MMPs associated permeability induced by DV infection. In particular, this study evaluated the effect of rhizome crude extracts of *Zingiber officinale* and pure [6]-gingerol on permeability induced by DV infection by determining MMP-2 and MMP-9 proteolytic activities through gelatin zymography and mRNA expression of MMP-2 and MMP-9 alongside those of TIMP-1 and TIMP-2 through quantitative real time RT-PCR in an *in vitro* model of DV-infection to provide new insights for the development of anti-dengue agents.

**1.2. Statement of the Problem**

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 host 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. DHF/DSS, the major life-threatening outcomes of severe dengue disease, are the consequences of plasma leakage in the vascular areas. 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. Thus, dengue remains one of the most pressing infectious diseases that has come to plague the tropical world with an unwavering annual mortality.

Considering the role of MMPs in vascular leakage during DHF/DSS, it is important to develop 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 valuable in controlling microvascular permeability-induced in severe dengue.

*Zingiber officinale* rhizome extracts and their biologically active compounds, including [6]-gingerol and shogaols were reported to be effective in *in vitro* models of various disease conditions by modulating the MMPs (MMP-2 and MMP-9) gene expression, protein expression and secretion (Yanti, 2011; Lee *et al*., 2008; Ling *et al*., 2010; Weng *et al*., 2010). Weng *et al*., 2010 demonstrated that mRNA expression of TIMP-1 was increased in an *in vitro* model of liver cancer after treatment with [6]-gingerol and [6]-shogaol (Weng *et*

*al*., 2010). However, the efficacy of *Zingiber officinale* rhizome extracts and its active compound [6]-gingerol in preventing vascular leakage by modulating MMPs and TIMPs cellular response in DHF/DSS has not been explored. This study is designed to fill these gaps.

**1.3. Objectives of the Study**

The general objective of this study is to investigate the effect of rhizome crude extracts of *Zingiber officinale* and pure [6]-gingerol on the proteolytic activity of MMP-2, MMP-9 and the mRNA expression of MMP-2, MMP-9, TIMP-1, TIMP-2 involved in vascular leakage in an *in vitro* model of dengue virus (DV3) infection.

Specifically, this study aims to:

1. determine the total phenolics and [6]-gingerol content of *Z. officinale* rhizome extracts using Folin–Ciocalteu reagent and High Performance Liquid Chromatography (HPLC);
2. determine the cytotoxic effects of *Z. officinale* rhizome crude extracts and pure [6]-gingerol on Vero cells and Madin-Darby Canine Kidney (MDCK) cells by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay;

3. determine the effects of *Z. officinale* rhizome crude extracts and pure [6]-gingerol on proteolytic activity of MMP-2 and MMP-9 in conditioned media collected from DV3 infected Vero cells using a gelatin-zymography assay;
4. determine the effects of *Z. officinale* rhizome crude extracts and pure [6]-gingerol on mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in DV3 infected Vero cells using quantitative real time RT-PCR after 48hrs of incubation;
5. determine the effects pure [6]-gingerol on mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in DV3 infected Vero cells using quantitative real time RT-PCR after 24hrs, 48 hrs and 72 hrs of infection;
6. demonstrate the effect of the *Z. officinale* rhizome crude extracts and pure [6]-gingerol on DV infection-induced permeability using an *in vitro* permeability assay.

**1.4. Significance of the Study**

There is a surging interest in the use of medicinal plants in developing countries because herbal medicines have been reported to be safe and without any adverse side effects. The development of new drugs from locally available

plant material may produce compounds with superior activity and provide cheap alternative medicines.

More than fifty years since its discovery, DV has sparked tremendous research efforts across the region for its control and treatment. At present, there is no effective drug, nor vaccine, against dengue infection and its disease manifestations. Certain herbal plants have been cited as promising effective remedies against dengue virus infection, but there is a scarcity of scientific evidence to support these claims. This study investigated the effect of *Z. officinale* rhizome crude extracts and pure [6]-gingerol on activity of MMP-2, MMP-9 and gene regulation of MMP-2, MMP-9, TIMP-1 and TIMP-2. Furthermore, the study will examine the effect on the cellular permeability in an *in vitro* model of dengue infection using *Z. officinale* rhizome crude extracts and [6]-gingerol which could lead to the isolation of potential compounds which can further be developed as novel drugs for the treatment of vascular leakage seen in dengue infection.

**1.5. Scope and Limitations of the Study**

This study made use of *Z. officinale* rhizomes (fibrous and nearly dry that was collected in between of 8-9 months after planting) as the source of plant extract. A total of four extracts extracted with various solvents of different polarities was tested for the total phenolic contents (TPC) using Folin-Ciocalteu

colorimetric methodology. The content of [6]-gingerol was determined by HPLC analysis. Only extracts as indicated by quality and quantity of TPC and [6]-gingerol were used for the bioassays. Pure [6]-gingerol was also used for the bioassays. The study was limited to use of pure [6]-gingerol based on its reported bioactivities.

The study did not include the isolation, identification and structural elucidation of the bioactive phytochemicals found in the extracts. Cytotoxic effects of the test extracts and pure [6]-gingerol on cultured cells was measured using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.

The different cell lines used in this study were obtained from the Research and Biotechnology division (RBD), St. Lukes Medical Center, Quezon City, Philippines which were procured from American Tissue Culture Collection (ATTC, USA). For the infection of cells this study was limited to DV3 (isolate SLMC-50). Strain SLMC-50 was chosen as it was derived from a clinical case of severe dengue and strains of serotype DV3 have been found to cause more severe illness in past infections in the Philippines. Culture-grown isolates of DV3 were obtained from the Dengue Biobank of the RBD, St. Luke's Medical Center, Quezon City, Philippines. *Aedes albopictus* C6/36 cells were used for DV propagation. *Mesocricetus auratus*, baby hamster, kidney cells (BHK-21) were used for titration of propagated virus by foci-formation assay. DV infectivity

titres were determined by foci formation assay. *Cercopithecus aethiops* (monkey) kidney cells (Vero) were infected with DV to induce MMP production and Madin-Darby Canine Kidney (MDCK) epithelial cells were used for permeability assays.

Virus free, concentrated conditioned media after DV infection was used for zymography and *in vitro* permeability assays. Effects of extracts and pure [6]-gingerol on MMP-2 and MMP-9 activity in the conditioned medium was measured using gelatin zymography. Real time RT-PCR was performed to determine the modulatory activity of extracts and pure [6]-gingerol on MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA levels respectively. The *in vitro* permeability assay was performed in a 24-well tissue culture plate with cell culture transwell inserts using extracts and pure [6]-gingerol. All the assays were done in triplicate unless otherwise mentioned.

**1.6. Definition of Terms**

**Dengue Fever:** An acute mosquito-borne viral illness of sudden onset that usually follows a benign course with headache, fever, prostration, severe joint and muscle pain, swollen glands (lymphadenopathy) and rash.

**Dengue Hemorrhagic Fever**: A severe form of the disease characterized by hemorrhage, shock, and encephalitis.

**Dengue Shock Syndrome:** A syndrome due to the dengue virus which is defined as the circulatory failure represented by a rapid and weak pulse and hypotension, in addition to hemorrhagic tendencies.

**Immunomodulatory:** having the ability to alter or regulate one or more immune functions.

**Matrix Metalloproteinases:** A family of zinc-dependent metalloendopeptidases that are involved in the degradation of extracellular matrix components.

**Matrix Metalloproteinase-2:** A secreted endopeptidase homologous with interstitial collagenase, but which possesses additional fibronectin-like domain and are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. It impairs tight junction proteins ZO-1, claudin-5 and occluding and adherens junction proteins VE-cadherin resulting in increased cellular permeability.

**Matrix metalloproteinase-9:** An endopeptidase that is structurally similar to matrix metalloproteinase-2. It degrades gelatin types I and V and collagen types IV and V. It also impairs the tight junctions and adherens junction proteins like MMP-2.

**Phytochemicals:** Biologically active chemicals found in plants that provide health benefits to humans.

**Plaque Assay:** An assay used for virus isolation and purification, and to determine infectivity and titers of viral isolates.

**Tissue Inhibitor of Metalloproteinase:** A family of proteins of around 200 residues that can inhibit enzymes of the metalloprotease type, for example collagenase, by binding to them.

**Tissue Inhibitor of Metalloproteinase-1:** A 207-aa; 28-kDa secreted glycoprotein growth factor which tightly complexes with and inhibits collagenase as well as connective tissue metalloendoproteases, involved in extracellular matrix degradation and connective tissue remodeling and it acts on MMP-1,-2,-3,-7,-8,-9,-10,-11,-12,-13 and -16.

**Tissue Inhibitor of Metalloproteinase-2:** Complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them by binding to their catalytic zinc cofactor. Known to act on MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-13, MMP-14, MMP-15, MMP-16 and MMP-19.

**1.7. List of Acronyms Used**

**DF** Dengue fever

**DHF** Dengue haemorrhagic fever

**DSS** Dengue shock syndrome

**DV** Dengue virus

**DV-1** Dengue virus serotype-1

**DV-2** Dengue virus serotype-2

**DV-3** Dengue virus serotype-3

**DV-4** Dengue virus serotype-4

**EGCG** Epigallocatechin-3-gallate

**HPLC** High Performance Liquid Chromatography

**MMP** Matrix metalloproteinase

**MMP-2** Matrix metalloproteinase-2

**MMP-9** Matrix metalloproteinase-9

**TIMP-1** Tissue inhibitor of metalloproteinase-1

**TIMP-2** Tissue inhibitor of metalloproteinase-2

**PBS** Phosphate-buffered Saline

**RT-PCR** Reverse transcription-polymerase chain reaction

**SDS** Sodium dodecyl sulfate

# CHAPTER 2

# LITERATURE REVIEW

## 2.1. General Description of Dengue

### 2.1.1. Dengue Incidence

Since its original isolation in 1943, DV has become an increasing concern worldwide (Mahy and van Regenmottel, 2007). DV circulates in tropical and subtropical regions of the world. Global dengue incidence has increased precipitously over the last five decades and severe dengue cases have also expanded (Gubler, 2011; WHO, 2013; WHO, 2012). Up to 3.6 billion people are estimated to be living in tropical and subtropical areas where the dengue viruses have the potential to be transmitted (Gubler, 2011; WHO, 2013; Ferreira, 2012). Global estimates vary, but regularly approximate 50 million to 200 million dengue infections, 500,000 episodes of severe dengue (DHF/DSS), and over 20,000 dengue related deaths occur annually (Shepard, 2011).

Dengue registered a 30-fold increase in disease incidence over the past 50 years. In the Americas, where the interruption of transmission occurred several decades ago as a result of an eradication campaign, many areas are now hyperendemic and outbreak are frequent in the Caribbean, Central America and

South America. In South East Asia, severe dengue is endemic in most of the countries including India, Indonesia and Sri Lanka. Although the number of dengue cases reported annually decreased to around 50,000 annually in 1999 and 2000 after an epidemic in 1998, the incidence of dengue has increased in the Western Pacific during the past decade. In 2010, the region reported 353,907 cases, including 1073 deaths. The incidence of dengue was highest in the Lao People's Democratic Republic, but most cases and deaths were reported from the Philippines (The Global dispatch, 2015).

**2.1.2. Dengue Symptoms and Classification**

There is a range of illness caused by infection by DV from unapparent infection to severe shock and death. The majority of DV infections are asymptomatic. DF, sometimes known as "breakbone fever", usually consists of an acute, self-limiting fever lasting 3-7 days (Rothman and Mathew, 2008). Other symptoms may include intense headache, painful joints and muscles, pain behind the eyes, and sometimes a rash (WHO, 2009). More severe symptoms may lead to vascular leakage, the hallmark of DHF. DHF symptoms include a fever lasting two to seven days, hemorrhagic tendencies, plasma leakage and thrombocytopenia (Teixeira and Barreto, 2009).

The World Health Organization has classified dengue virus infection into four grades. Subjects with Grade I display a fever accompanied by other

nonspecific symptoms. This is considered either DF or DHF. Grade II subjects have DHF, and suffer from spontaneous hemorrhagic manifestations. Grade III subjects show circulatory failure with a rapid yet weak pulse and a diminished blood pressure (20 mmHg or less). Grade IV subjects suffer from profound shock and undetectable pulse and blood pressure (WHO, 2009). DHF may lead to DSS. DSS is defined as circulatory failure represented by a rapid and weak pulse and hypotension, in addition to hemorrhagic tendencies (Teixeira and Barrito, 2009).

A new classification has been developed recently by WHO for clinicians to make better decisions regarding patient care: dengue (D), dengue with warning signs (DW), and severe dengue (SD) (WHO, 2010). SD 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 DSS via severe plasma leakage (Lei, 2008).

According to the WHO, DHF causes 500,000 hospitalizations annually, mostly in children. Of the 890,000 reported cases of dengue infection in 2007, 26,000 of these were classified with DHF. Although patients classified with DHF have a 2.5% mortality rate, this number can rise to approximately 20% in regions where proper medical treatment is not available (WHO, 2009).

### 2.1.3. Dengue Prevention and Treatments

Current disease prevention is limited to eradicating the mosquito vector via insecticides and by removing structures containing standing water (old tires, gutters) to decrease the spread of infected mosquitoes. Although some viral diseases can be prevented by vaccines, recent attempts to develop an effective, safe, economical dengue virus vaccine has been unsuccessful because of the concerns about antibody-dependent enhancement (ADE) and increased severity of secondary infections. Current treatments for DHF patients include controlling febrile symptoms and replenishing plasma after plasma leakage.

### 2.1.4. Dengue Virus Serotypes

Four different serotypes of DV have been isolated and studied. These serotypes are designated DV1, DV2, DV3, and DV4. Although the serotypes share 60-80% homology with each other, they remain structurally different (Mahy and van Regenmottel, 2007). A primary infection with any one of the four serotypes by means of a mosquito bite leads to lifelong immunity to that serotype. Secondary infection requires productive infection by a different DV serotype, heterologous from the primary infecting serotype, and leads to 90% of all severe cases, increasing the risk of DHF by 15-80 folds. Thus, one antibody type only provides partial short-term protection against other DV serotypes (Sabin, 1952). A challenge for any vaccine under development is protection against DHF.

Furthermore, immunizing against a single serotype, or incomplete vaccination against any single serotype, may lead to increased risk of a more severe infection (Rothman, 2004). Although DV2 is known to be more lethal than other serotypes (Goel *et al*., 2004), some studies have revealed that primary infection with DV1 or DV3 always results in more dangerous disease than infection with DV2 or DV4 (Guzman and Isturiz, 2010; Tang *et al*., 2012).

**2.1.5. Pathogenesis of DF, DHF and DSS**

The mechanism of severe dengue infections are still not completely understood but are likely to be multifactorial. The genetic background of the host influences the way that the immune response reacts to DV infection. During the feeding of mosquitoes on humans, DV is presumably injected into the bloodstream, with spill over in the epidermis and dermis, resulting in infection of immature Langerhans cells (epidermal dendritic cells [DC]) (Limon-Flores *et al*., 2005; Wu *et al*., 2000), and keratinocytes (Limon-Flores *et al*., 2005). Infected cells then migrate from site of infection to lymph nodes, where monocytes and macrophages are recruited, which become targets of infection. Consequently, infection is amplified and virus is disseminated through the lymphatic system. As a result of this primary viremia, several cells of the mononuclear lineage, including blood-derived monocytes (Durbin *et al*., 2008), myeloid DC (Boonnak *et al*., 2008; Ho *et al*., 2004; Kwan *et al*., 2005), and splenic and liver macrophages

(Blackley *et al*., 2007; Kou *et al*., 2008; Jessie *et al*., 2004) are infected. DV has also been shown to have tropism for circulating mononuclear cells in blood and for cells residing in the spleen, lymph nodes, and bone marrow of infected AG129 mice (Kyle *et al*., 2007).

When infecting the target cells, the envelope protein aids in the attachment of the virus to a target cell (Seema and Jain, 2005). Once attached to the host cell, viral infection occurs via endocytosis, then uncoating and expression of the viral genome (Marsh and Helenius, 1989). This is followed by the assembly of new virus in the cell, release of the virions, and attachment of the virus to receptors on another uninfected cell (Marsh and Helenius, 1989).

During primary DV infection, inflammatory cytokines are released triggering an adaptive immune response including T cells, NK cells, and B cells responding to the virus. Naïve T cells that show specificity for the invading serotype expand and mount a response. As part of this response, T cells also release inflammatory mediators (Buchy *et al*., 2007). Primary infection tends to last three to seven days, rarely requires hospitalization, and is not associated with severe symptoms (Halstead, 1980).

In the 1960's, a study concluded that greater than 85% of children suffering from DHF in Bangkok showed high dengue serotype cross-reactive antibody titers (Halstead *et al*., 1970). Subsequent research confirmed that DHF is substantially more common in secondary infections.

*Antibody-Dependent Enhancement*

Many theories contribute to the understanding of pathogenesis of secondary infection. One theory proposed is antibody-dependent enhancement (ADE). According to this theory, non-neutralizing antibodies bound to the DV virus are taken up by cells containing the Fc receptor. This viral entry is mediated by antibodies specific for DV from the primary infection (Gollins and Poterfield, 1969). Because antibody-bound virus attaches to the Fc receptors more efficiently than in a primary infection, there is an enhancement of penetration and fusion of the virion envelope with the membrane (Halstead, 1988). ADE increases the number of infected cells which then causes increased viral load and increased vascular permeability (Halstead, 1989). IgG type antibodies specific for E and NS1 antigens expressed on the surface of the infected cell are the primary antibody response to a secondary infection (Halstead, 1988). Results have shown evidence of ADE *in vitro*, however due to difficulties with animal models, *in vivo* results are limited (Rothman, 2009). Non-human primate models have been used to study antibody responses during secondary dengue infection. These studies have shown an increase of viremia in primates receiving passive immunity with DV-specific antibody (Halstead, 1979; Goncalvez *et al*., 2007). Although this provides *in vivo* evidence for ADE, the primate model is still controversial as it does not correspond with human pathogenesis.

*T Cell-Mediated Immunopathogenesis and Cytokine Storm*

Another model to explain the observed plasma leakage during secondary infection is referred to as the T cell-mediated immunopathogenesis. T-cell mediated immunopathogenesis causes a cytokine storm. According to this model, a more rapid reactivation of memory DV-specific T cells due to an increased presentation of antigens on infected cells leads to a stronger secondary infection. The cross-reactive memory T cells still present from the primary infection have a lower affinity to the current serotype and become activated after the secondary infection. These memory T cells out compete naïve T cells for the infecting serotype, and therefore alter the T cell response to result in a higher activation of T cells during a severe disease, a more rapid cytokine production, and an expansion of the lower avidity memory T cells.

The increased over-production of cytokines may lead to plasma leakage. A T cell response to DV infection includes the secretion of Th1 cytokines, such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), lymphotoxin A (LTA), macrophage inflammatory protein 1β (MIP-1β), and IL-2. Small amounts of the Th2 cytokine, IL-4, are also secreted (Kurane and Ennis, 1989). IFN-γ activates monocytes and macrophages, and has been shown to upregulate the expression of Fcγ receptors and HLA (Gagnon *et al*., 1999; Goncalvez *et* al., 2007). IFN-γ is elevated in both DF and DHF patients (Kurane *et al*., 1991; Hober *et al*., 1993). Higher peak levels of IFN-γ were found in patients with DHF (Green *et al*., 1999).

TNF-α is produced by monocytes and macrophages, and has been shown to produce an increase in vascular permeability (Kurane and Ennis, 1988). IL-2 is a cytokine produced by Th1 12 cells which activate T and B cells, and NK (Natural Killer) cells. IL-2 has been associated with capillary leak syndrome and thrombocytopenia (He *et al*., 1995).

IL-2 is highly elevated in both DF and DHF patients. The soluble IL-2 receptor (sIL-2R) displayed much higher levels in DHF patients compared to DF patients (Boonpucknavig *et al*., 1979). IL-8 is also believed to play a role in DHF. Levels of IL-8 are elevated in DHF patients compared to DF patients (Raghupathy *et al*.**,** 1998). DV has been shown to increase IFN-γ, TNF-α, LTA, MIP-1β, IL-2, and IL-8 secretion leading to an over-production of these cytokines and severe disease, particularly in DV-2 and DV-3 infections (Leitmeyer *et al*., 1999; Vaughn *et al*., 200; Nogueira *et al*., 2002). Higher levels of IL-13, IL-18, IL-1β, IL-6, and IL-10 were also observed in DF and DHF patients (Hober *et al*., 1993; Green *et al*., 1999; Mustafa *et al*., 2001). IL-10 is an anti-inflammatory cytokine that displayed higher levels in DHF patients than DF patients (Green *et al*., 1999). The systemic over-production of cytokines during a secondary infection of DV causing inflammation and plasma leakage results in a “cytokine storm”. In addition, this model also incorporates the previous model, as non-neutralizing antibodies present after a primary infection are thought to heighten uptake of the virus into

antigen presenting cells. This causes a greater T cell activation, further increasing the likelihood of plasma leakage (Rothman and Mathew, 2008).

*Complement*

Reduction in the levels of complement components have been described in patients with severe dengue, suggesting that complement activation may have a role in the pathogenesis of severe disease (Malasit, 1987) Excessive complement activation at endothelial surfaces contributes to the vascular permeability observed in severe dengue (Avirutan *et al*., 2006). Dengue virus NS1 protein attenuates classical and lectin pathway activation of complement by directly interacting with C4. It promotes degradation of C4 to C4b and by this mechanism, NS1 is suggested to protect DV from complement-dependent neutralization in solution (Avirutan *et al*., 2010). It is plausible that the low levels of complement observed in severe dengue are merely a marker of a severe systemic disease rather than an indicator of their role in capillary permeability.

**2.1.6. Dengue Virus**

*Structure*

DV has a positive-sense single-stranded RNA genome of about 11,000 nucleotides in length with a 5’ 7-methylguanosine cap and no polyadenylic acid.

On either side of the genomic open reading frame are a 5' non-coding region (NCR) of 100 nt and a 3' NCR of 400-700 nt consists of hairpin loops necessary for RNA translation and virus replication (Figure 2.1). The 3' NCR contains a 3' stem loop containing essential virus-specific and host-specific functional regions. The 3' NCR enhances the translation of a reporter mRNA containing this structure. Upstream from the 3' stem loop resides the CS1 region. The CS1 is a twenty-five nucleotide region which pairs with a complementary sequence in the 5'CS region (Lindenbach *et al*., 2007).

Translation of dengue virus RNA creates a single polyprotein that is co- and post-translationally processed by viral and cellular proteases. Cleavage of the polyprotein yields 3 structural proteins, and 7 non-structural (NS) proteins in the order: (5') capsid (C), membrane (prM), envelope (E), non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (3')(Figure 2.1). Host cell enzymes (signal peptidase or furin) or viral NS2B/NS3 cleave this polyprotein to yield the individual structural and nonstructural proteins (Jacobs *et al*., 2007).

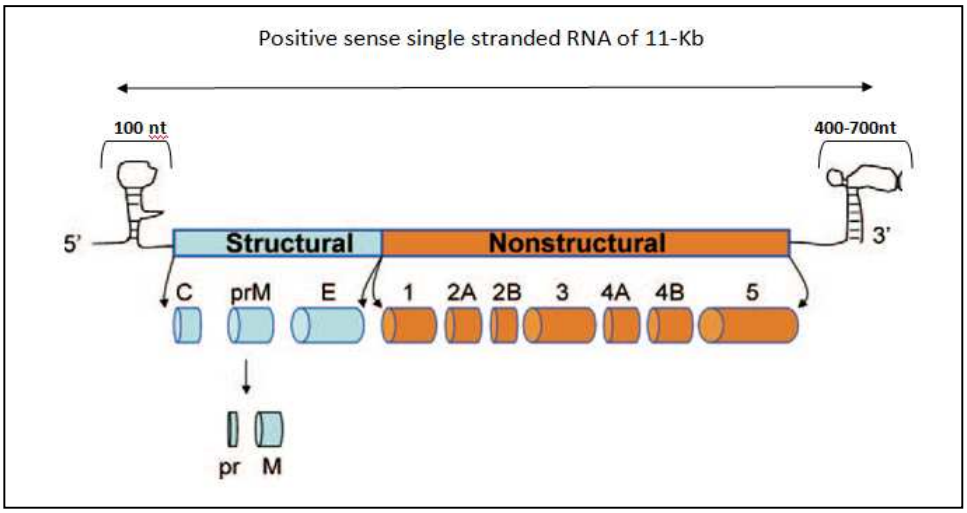



**Fig. 2.1 The dengue viral genome.** Noncoding regions with their terminal structures are indicated by black lines. The single open reading frame encodes a poly-protein that is processed by the viral NS2B-NS3 protease and cell proteases to the mature viral proteins. Structural proteins are C, prM, and E. Nonstructural proteins are 1, 2A, 2B, 3, 4A, 4B, and 5. Figure modified from Noisakran and Perng, (2008).

### 2.1.7. DV Classification

DV is a member of the taxonomic family Flaviviridae, genus *Flavivirus* and is transmitted through the mosquito vector *Aedes aegypti*. Flaviviruses are enveloped, positive-stranded RNA viruses. DV shares the *Flaviviridae* classification with eight other viruses with similarities in both structural and non-structural proteins. Other family members include: Aroa virus, Japanese encephalitis, Kolobera virus, West Nile virus, and Yellow fever virus. DV also belongs to a larger heterogeneous group of viruses transmitted by insect vectors, called arboviruses. For these viruses, the transmission to vertebrate

hosts is dependent on arthropod vectors, which for DV is *Aedes aegypti.* Currently about two thirds of the world population live in areas with *Aedes aegypti* (Pinheiro and Corber, 1997).

**2.1.8. DV Replication**

The DV virion is a spherical enveloped virus that has a diameter approximately 50 nm consisting of the 3 structure proteins (C), (M), (E), and the virus RNA. Virions enter the host cell by means of receptor-mediated endocytosis (Clyde and Harris, 2006). The viral envelope protein binds to corresponding receptors on the host cell membrane to mediate this process. The E protein mediates the fusion of the viral membrane and plasma membranes. Pre-lysosomal endocytic compartments have a low pH which causes the viral envelope to fuse with the host cell membranes. The nucleocapsid is released into the cytoplasm, where the RNA genome is separated from the capsid proteins with which it is complexed. Upon translation, a single polyprotein is made which is then cleaved into ten different products by viral and host proteases (Whitehead *et al*., 2007). The flavivirus genome is replicated on intracellular membranes and genomic length negative or antisense strand RNA is synthesized, then serves as a template for positive strand RNA synthesis. The positive or sense strand RNA when base-paired with the negative strand is called the replicative form (RF) (Uchil and Satchidanandam, 2003). The RF serves as the

template for positive RNA synthesis and this is called the replicative intermediate (RI). As positive strand RNA is released from the RI it is either translated by ribosomes or packaged into virions in the rER. The viral glycoproteins undergo maturation in the Golgi, becoming glycosylated; prM is cleaved by furin, and the E protein homodimerizes. At this point the M and E proteins rearrange on the virion surface yielding mature infections virions (Mukhopadhyay *et al*., 2005).

Finally, the mature virion exits the infected cell which has a smooth surface, with E proteins laying in pairs parallel to the virion surface. The E glycoprotein can be divided into three structural domains which either aid in cell attachment, fusion, or targeting by protective antibodies. These are the central domain, the dimerization domain which presents a fusion peptide, and the receptor-binding domain (Kuhn *et al*., 2002).

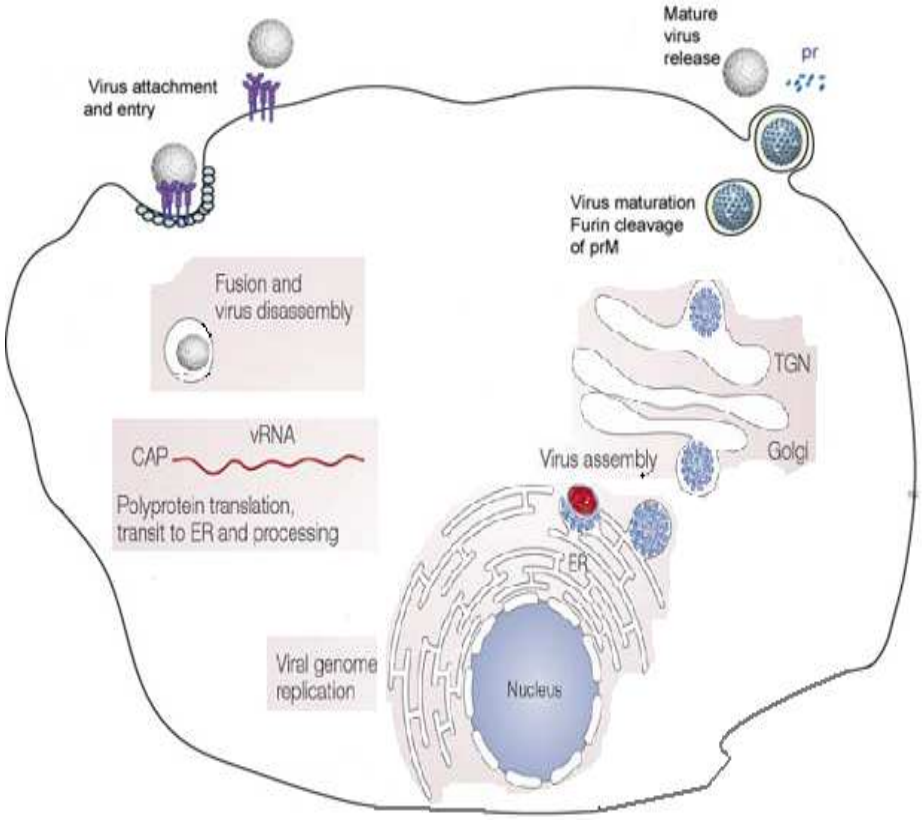



**Fig. 2.2 The dengue virus life cycle within the cell.** The virus binds the cell and its envelope fuses with the host cell membranes, releasing the genome into the cytoplasm. Translation produces a single polyprotein which is cleaved into ten different products. New virions bud into the endoplasmic reticulum and exit the host cell by means of exocytosis. Figure modified from Mukhopadhyay *et al*., (2005).

## 2.2. The Vascular Endothelium

Microvascular plasma leakage is the hallmark of DHF/DSS. The precise molecular mechanisms leading to the microvascular leakage are currently unknown, but disruption of the endothelial cell barrier may be a critical step.

Thus, it is essential to understand the basic structure of the vascular endothelium.

### 2.2.1. Basic Structure of the Vascular Endothelium

Endothelial cells (EC) adhere to each other through several major junctions: (a) tight junctions, (b) adherens junctions, and (c) gap junctions (Fig. 2.3). Integrins on the cell surface anchor cells to the extracellular matrix (ECM). The interaction of the ECM with integrins generates signals that inhibit EC proliferation and migration, but stimulate cell-cell and cell-ECM adhesion (Mehta and Malik, 2006). Together, these intercellular and ECM interactions form the endothelial barrier (Bazzoni and Dejana, 2004; Mehta and Malik, 2006). In models studying vascular endothelium permeability, the disruption of adherens junctions leads to permeation of otherwise impermeable (or slightly impermeable) molecules (Bazzoni and Dejana, 2004; Mehta and Malik, 2006). Thus, proteins involved in the adherens junctions, including the vascular endothelial cell specific transmembrane protein VE-cadherin, are often examined. Junctional adhesion proteins are also responsible for connecting cytoskeletal and signaling proteins through their cytoplasmic tails, thus allowing for anchoring to actin microfilaments and intracellular signal transduction (Dejana, 2004). This association provides for stabilization of the adherens

junction and for the regulation of junctional permeation (Bazzoni and Dejana, 2004; Dejana, 2004).

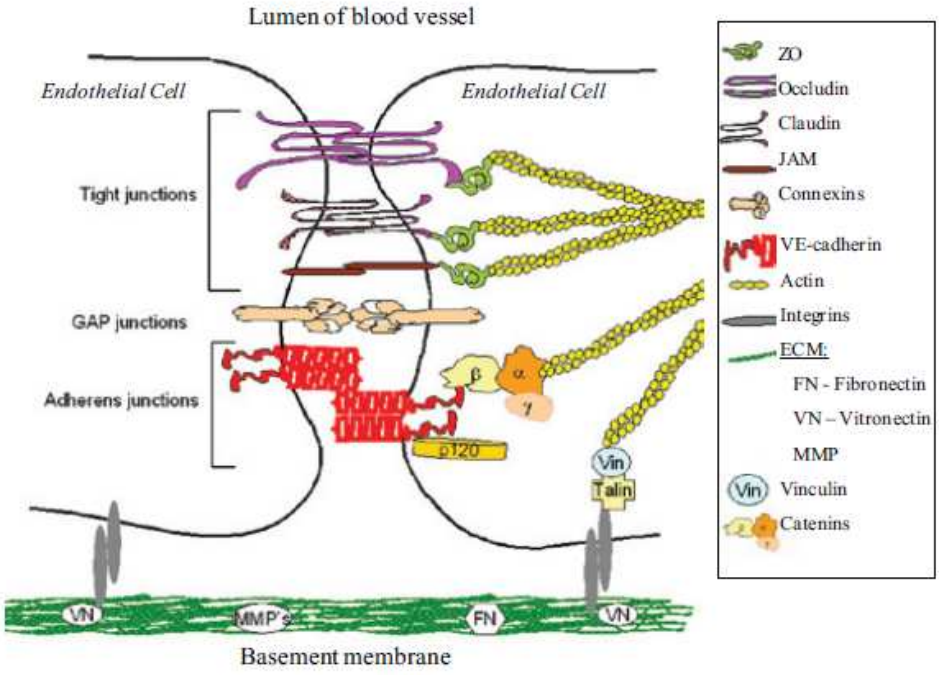



**Fig. 2.3 Structural organization of endothelial cell intercellular and matrix interactions.** Tight junctions are located on the apical side of the EC barrier with their adhesion properties mediated by occludins, claudins, and junctional adhesion molecules (JAMS). Zona occluden-1 protein (ZO-1) links tight junction proteins to the cytoskeleton, thus stabilizing the tight junction. Gap junctions are composed of connexin complexes called connexons. Adherens junctions are formed through cadherin-cadherin protein interactions. Cadherin association with α-, β-, and γ-catenins allow for anchoring to actin microfilaments. Integrins on the cell surface anchor cells to the extracellular matrix proteins. Figure adapted from Mehta and Malik, (2006).

**2.3. Endothelium Damage Caused During Dengue Hemorrhagic Fever**

It is unclear how the vascular endothelium is affected during DHF/DSS or what triggers vascular leakage, though direct and indirect mechanisms of vascular leakage have been proposed.

**2.3.1. Endothelium Damage Caused Directly by Dengue Virus**

DV-infection induced apoptosis of EC leading to loss of integrity of vascular endothelial barrier and the consequent permeability, observed in DHF/DSS (Liew and Chow, 2004). Secretion of inflammatory cytokines by DV-infected endothelial cells is another proposed trigger of vascular leakage. *In vitro*, infected HUVEC and human dermal microvascular endothelial cell line (HMEC-1) monolayers produce cytokines like IL-6 and IL-8, which can induce microvascular leakage (Huang *et al*., 2000; Talavera *et al*., 2004). Furthermore, IL-8 recruit lymphocytes, eosinophils, and neutrophils to the site of its production. Therefore, it is likely that EC may recruit effector cells to the site of infection by producing chemokines following DV infection, thus exacerbating local inflammation and induce a cascade of events leading to microvascular permeability.

**2.3.2. Endothelium Damage Caused by Indirect Mechanisms**

Microvascular plasma leakage may occur independently of direct DV infection of EC. A key observation in DHF/DSS is that symptoms of severe

dengue, appear as the patient's condition suddenly worsens around the time of defervescence, and clearance of viraemia (Whitehead *et al*., 2007). Thus, a strong inflammatory response may cause damage to EC barrier function. Activated immune cells may play a role in the vascular leakage observed in DHF/DSS through the secretion of inflammatory cytokines. Elevated levels of TNF- α, IL-6, IL-1β, IL-8, IFN- γ and MCP-1 have been documented in the serum of severe dengue patients (Lee *et al*., 2006; Pang *et al*., 2007). The possible contributors of these cytokines include DV-infected or activated monocytes/macrophage, T cells, B cells, and even mast cells. Monocytes/macrophages, infected *in vitro* with DV alone or under ADE conditions have been shown to release factors into culture medium that increase permeability of HUVEC barrier (Carr *et al*., 2003; Lee *et al*., 2006). These factors include, but are not limited to: IL-1, IL-6 (Lin *et al*., 2002), IL-8 (Bosch *et al*., 2002), and MCP-1 (Lee *et al*., 2006). Dengue-specific T cells may also contribute to cytokine production and vascular leakage. Reports show that patients with secondary heterotypic dengue infections express many dengue-specific CD8+ T that produce elevated levels of cytokines, including IFN-γ and TNF-α (Mongkolsapaya *et al.*, 2003; Mongkolsapaya *et al*., 2006). In addition, isolated, human primary B cells can also be infected with DV directly or under ADE conditions *in vitro*, and these infected B cells secrete IL-6 and TNF- α (Lin *et al*., 2002).

Matrix degrading metalloproteinases (MMPs), zinc-dependent endopeptidases, together with inflammatory cytokines *e.g.*IL-1, IL-6, TNFα are believed to contribute largely toward the progression of severe pathology for dengue infection (Martina *et al*., 2009; Nielsen, 2009). MMPs produced by Dengue virus-infected cells have an important role in inducing *in vitro* endothelial cell monolayer permeability and in *in vivo* mouse model (Luplertlop *et al*., 2006). It has previously been found that following DV infection, 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 (Luplertlop and Misse, 2008).

DV NS1 protein may be another indirect trigger of vascular leakage. NS1 is not packaged into dengue virions, but exists in three forms: soluble NS1, membrane-bound NS1, and ER-residing NS1 which colocalizes with the viral replication complex (Clyde *et al*., 2006). Anti-NS1 antibodies are thought to cross-react with human EC; stimulate EC into producing IL-6, IL-8, and MCP-1 (Lin *et al*., 2005); and induce EC apoptosis (Lin *et al*., 2003). However, another interpretation of these results is that antibodies to NS1 recognize NS1 bound to EC through interactions with heparan sulfate and chondroitin sulfate E and that these interactions contribute to vascular leakage (Avirutnan *et al*., 2007). Yet another role for NS1 in DHF/DSS is as an activator of complement. Soluble or

membrane-bound NS1, in combination with NS1-specific antibodies, was shown to activate complement. Furthermore, plasma levels of NS1 and terminal complement SC5b-9, correlated with the severity of DV illness (Avirutnan *et al*., 2006). Activation of complement, and subsequent generation of small complement factors C3a, C4a, and C5a, may also lead to the increased vascular permeability observed in DHF/DSS.

**2.3.3. Thrombocytopenia**

Thrombocytopenia is a recurrent observation in DHF/DSS and is thought to be associated with hemorrhage (Lin *et al*., 2001). The major causes of thrombocytopenia are believed to be peripheral consumption and destruction of platelets (Nimmannitya, 1999). Destruction of these platelets may be caused directly by DV or through binding of DV specific antibodies to infected or uninfected platelets (Noisakran and Perna, 2008) been observed in sera from patients with DV. Anti-NS1 antibodies also bind human platelets to facilitate macrophage platelet binding and possibly phagocytosis, complement-mediated lysis of platelets, and activation of platelets (Sun *et al*., 2007). Furthermore, treatment of mice with these anti-NS1 antibodies induced thrombocytopenia. It is thus possible that antibodies to NS1 are linked to thrombocytopenia. In the presence of complement, these DHF/DSS patient sera induced platelet lysis (Lin *et al*., 2001). DHF sera have also been shown to significantly enhance platelet-

macrophage engagement (Sun *et al*., 2007). These studies suggest that factors in DHF/DSS sera lead to enhanced clearance of platelets by macrophage or phagocytes.

### 2.4. General Description of *Zingiber officinale*

#### 2.4.1. Botany and Distribution of *Zingiber officinale*

Zingiberaceae, known as ginger family, is a perennial herb plant, terrestrial and rich in aromatic compounds. It has been noted that there are 50 genera and 1300 species of Zingiberaceae plants. Most of them like *Curcuma* sp., *Kaempferia* sp., *Alpinia* sp. and *Zingiber officinale* (ginger) are utilized worldwide as both culinary and medicinal use (Park and Pezzuto, 2002). It is one of the oldest and most commonly used spices. Ginger for these uses is obtained from the tuberous rhizome, or underground stem of the plant. It is also called root ginger to distinguish it from other things which share the name ginger (Rehman *et al*., 2011).

*Z. officinale* is herbaceous rhizomatous perennial, reaching up to 90 cm in height under cultivation. Rhizomes of this herb are aromatic, thick lobed, pale yellowish, bearing simple alternate distichous narrow oblong lanceolate leaves. The herb develops several lateral shoots in clumps, which begin to dry when the plant matures. Its leaves are long and 2 - 3 cm broad with sheathing bases, the

leaf blade is gradually tapering to a point. The flowers are rare, rather small, calyx superior, gamosepalous, three toothed, open splitting on one side, corolla of three subequal oblong to lanceolate connate greenish segments (Kawai, 1994).

**Fig. 2.4 Photograph of *Zingiber officinale* rhizome**

### 2.4.2. Taxonomy of *Zingiber officinale*

*Zingiber officinale* is a species of family *Zingiberacea* known by the common names *Luya* in the Philippine and also known as Ginger. Below is the taxonomic hierarchy of *Zingiber officinale.*

**Domain :** Eukaryota

**Kingdom :** Plantae

**Subkingdom :** Viridaeplantae

**Phylum :** Tracheophyta

**Subphylum :** Euphyllophytina

**Infraphylum :** Radiatopses

**Class :** Magnoliopsida

**Order :** *Zingiberales*

**Family :** *Zingiberacea*

**Genus :** *Zingiber*

**Species:** *officinale*

*Zingiber officinale*

### 2.4.3. Uses of *Zingiber officinale*

*Zingiber officinale* is used for nutritional and in folkloric medicine. As nutritional purpose it is a prominent condiment in Filipino cuisine and used as flavoring for confectioneries, ginger ale, ginger beer, ginger champagnes, and other beverages. Salabat, a native beverage, is prepared from the rhizomes. Ginger taken with rocksalt before meals is cleansing to the tongue and throat and increases the appetite. In Malaya fresh ginger is an important ingredient in curry.

Similarly as folkloric purpose, pounded rhizome, alone or mixed with oil is used as revulsive and anti-rheumatic in the Philippines. Tincture of dried rhizome prepared with 70% alcohol is applied on superficial cuts and wounds; or, juice

from fresh rhizome is used as antiseptic. As digestive aid and for flatulence and tympanism, decoction of the rhizome is drunk as tea. For sore throat and hoarseness, warm decoction of the rhizome is drunk as ginger tea (salabat) or a piece of small rhizome is chewed for the same. Rhizome is used as cough remedy, rubifacient, carminative and diuretic and also used for hangovers. Chewing ginger is said to diminish biliousness and delirium, relieve sore throat, hoarseness and aphonia, and it increases the flow of saliva. In Indo-China, cataplasm used for furuncles and when mixed with oil is anti-rheumatic. Rhizomes of ginger are also used for tuberculosis, general fatigue and uterine affections. In Perak, rhizomes are used as vermifuge. Similarly in the Antilles powdered rhizome is used as revulsive for pleuritis. It is used for inflammation and rheumatism in Ayurvedic medicine. In India, it is used as carminative adjunct along with black pepper and long pepper. Pulverized fresh ginger is used for baldness and vitiligo in Chinese folk medicine. Likewise Juice from fresh root is used for treatment of burns (Stuart, 2011).

### 2.4.4. Phytochemicals in *Zingiber officinale*

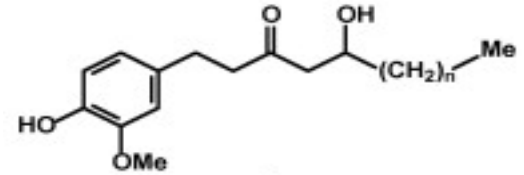
The active ingredients in *Zingiber officinale* are in volatile oil comprising approximately 1 - 3% of its weight. There are more than fifty components of the oil have been characterized and these are mainly monoterpenoids [β-phellandrene, cineole, curcumene, (+)-camphene, borneol geraniol, citral,

terpineol,] and sesquiterpenoids [α-zingiberene (30–70%), β-sesquiphellandrene (15–20%), β-bisabolene (10–15%), *ar*-curcumene, (*E*-*E*)-α-farnesene, , zingiberol] (Langner *et al*., 1998; Evans, 2002).

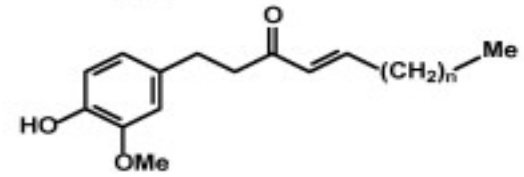
Gingerols, are responsible for the pungency of fresh ginger. [6]-gingerol is the most abundant one, though lesser amounts of other gingerols are also present. However, shogaols (like [6]-shogaol), which are dehydrated forms of gingerols are responsible for the pungency of dry ginger. Jolad *et al*., (2004) also demonstrated that the thermal degradation of gingerols formed gingerone, shogaols, and related compounds.

Organically-grown fresh ginger was examined and 63 compounds were identified, of which thirty one components had been previously reported and 20 were thus far unknown compounds. The identified components were gingerols, shogaols, paradols, acetyl derivatives of gingerols, gingerdiols, mono- and di-acetyl derivatives of gingerdiols, 3-dihydroshogaols, dihydroparadols, 1-dehydrogingerdiones, diarylheptanoids, and methyl ether derivatives of some of these compounds. [4]-, [6]-, [8]-, [10]- and [12]-shogaol, 4]-, methyl [6]- and methyl [8]-shogaol were characterized. [6]-, [7]-, [8]-, [9]-, [10]-, [11]-, and [13]-paradols, methyl [6]-paradol were also detected in the fresh ginger In addition to [6]-gingerol, [4]-, [7]-, [8]-, and [10]-gingerol, methyl [4]-gingerol and methyl [8]-gingerol also were identified (Jolad *et al*., 2004)

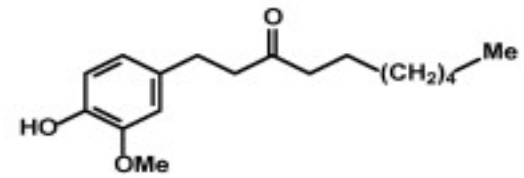
Jolad *et al.* (2005) also examined commercially processed dry ginger and identified a total of one hundred and fifteen compounds, of which 88 were reported. Of these, 45 had been recorded previously for fresh ginger (Jolad *et al.*, 2004) and 31 were new compounds, which included [6]-isoshogaol, methyl [6]-isogingerol and methyl [8]-paradol. The remaining 12 constituents had been isolated previously by other workers. [6]-, [8]-, [10]- and [12]-gingerdiones were detected and they had not been previously reported in fresh white and yellow gingers (Fig. 2.5).



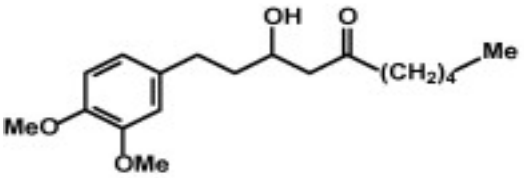

n = 4 [6] - gingerol **1**
n = 2 [4] - gingerol **3**
n = 5 [7] - gingerol **4**
n = 6 [8] - gingerol **5**
n = 8 [10] - gingerol **6**



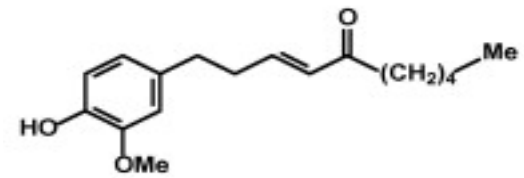

n = 4 [6] - shogaol **2**
n = 2 [4] - shogaol **7**
n = 6 [8] - shogaol **8**
n = 8 [10] - shogaol **9**
n = 10 [12] - shogaol **10**



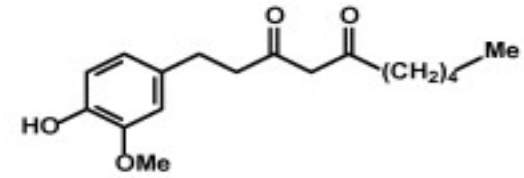

[6] - paradol **11**



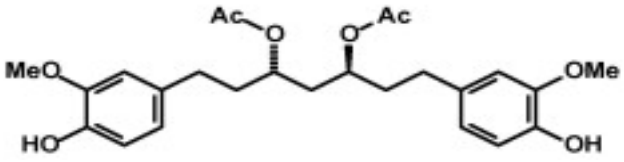

methyl [6] - isogingerol **12**




[6] - isoshogaol **13**




[6] - gingerdione **14**




(3*S*,5*S*) - 3,5 - diacetoxy - 1,7 - *bis* (4-hydroxy-3-methoxy) - heptane **15**

**Fig. 2.5 Major chemical constituents of ginger.** [6]-gingerol is the most abundant one, though lesser amounts of other gingerols are also present. However, shogaols (like. [6]-shogaol), are dehydrated forms of gingerols. (Adapted from Ali *et al*., 2008).

**2.4.5. *Zingiber officinale* (Ginger) Bioactivity**

It is known and valued that ginger extracts and its biologically active compounds such as gingerols, shogaols, paradols and zingerone exert immuno-modulatory, antiapoptotic, anti-tumourigenic, anti-inflammatory, antihyperglycaemic, anti-hyperlipidaemic, antioxidant and anti-emetic activities (Rasmussen, 2011). Several studies have shown that ginger possesses anti-cancer, anti-microbial, anti-inflammatory, and antioxidant effect (Liu *et al*., 2012; Bellik, 2014; Rasyidah *et al*., 2014; Hu *et al*., 2012; Lee *et al*., 2012; Rahman *et al*., 2011).

Phenylpropanoid-derived compounds such as [6]-gingerol and shogaols are biologically active constituents present in ginger and 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. For the first time Lee *et al*., (2008) reported that [6]-gingerol repressed the metastasis of MDA-MB-231 breast cancer cells with an accompanying reduction in activities and expressions of MMP-2 and MMP-9. Moreover they observed that the amount of MMP-2 protein was reduced and MMP-9 protein level was unchanged in culture supernatant following treatment with [6]-gingerol.

PMA-stimulated MDA-MB-231 cell invasion was inhibited by treatment with Shogaols (6-, 8- and 10-shogaol) with an accompanying reduction in MMP-9

gene activation, protein expression and secretion in a dose-dependent manner (Ling *et al*., 2010)

The data of Weng *et al*., (2010) suggested that [6]-gingerol and [6]-shogaol regulate MMP-9 and TIMP-1 activity and expression exerting anti-invasive activity against hepatoma cells. Indeed, MMP-9 activity was decreased, whereas the expression of TIMP-1 was increased in PMA-treated HepG2 cells and PMA-untreated Hep3B cells with 6-shogaol and 6-gingerol.

Similarly the data of Yanti (2011) showed that extracts of selected Zingiberaceae like *Curcuma xanthorrhiza*, *C. mangga C. aeruginosa, , C. longa*, *Zingiber officinale, Alpinia galangal* and *Kaempferia galangal* effectively decreased the MMP-9 secretion, mRNA and protein expression in LPS-induced *in vitro* model of artherosclerosis. Ginger extracts have also been shown to have antiviral activity against human syncytial respiratory virus and may activate macrophages in order to inhibit influenza virus infection (Chang *et al*., 2013; Imanishi *et al*., 2006). However little is known about the specific role of [6]-gingerol in viral infection.

The researchers attempted to identify novel lead compounds with antiviral effect, methanol and aqueous extracts of 8 medicinal plants in the Zingiberaceae family found that HIV-1 protease was strongly inhibited by the methanol extract of *Alpinia galanga*. This extract also inhibited HCV and HCMV proteases, but to a lower degree. HCV protease was most efficiently inhibited by

the extracts from *Zingiber officinale*, with little difference between the aqueous and the methanol extracts. Many of the methanol extracts inhibited HCMV protease, but the aqueous extracts showed weak inhibition (Sookkongwaree *et al*., 2006).

[6]-gingerol has been found to exhibit antioxidant activity as determined by inhibition of phospholipid peroxidation induced by the $FeCl_3$-ascorbate system (Aeschbach *et al*., 1994) and confirmed in many *in vitro* and *in vivo* system (Masuda *et al*., 2004; Kikuzaki *et al*., 1994). Gingerol has also been found to inhibit platelet aggregation and formation of prostaglandin and leukotriene (Srivastava, 1986; Guh *et al*., 1995).

Ginger has been described as great heart tonic. It helps in preventing various heart diseases by reducing blood clotting that can lead to plaque formation or thrombosis. It can also open the blockage in the blood vessels thus decreasing peripheral vascular resistance and hence blood pressure. It also may help to lower high cholesterol making the heart healthy (Akoachere *et al*., 2002).

The essential oil of *Zingiber officinale* has antimicrobial and antioxidant activity (Bellik, 2014). Mahady, (2003) demonstrated that methanol extract of dried ginger inhibited 19 strains of *Helicobacter pylori in vitro*.

Earlier finding has reported that 6-gingerol showed its anti-tumoral activity through induction of ROS which is also known to trigger activation of p53 and the cell cycle arrest and apoptosis (Yang *et al*., 2010). Another important and

first finding showed that *in vitro* and *in vivo* anticancer activity of whole GE for the management of prostate cancer (Karna *et al*., 2012). *Z. officinale* also shows a role in suppressing the expression of LPS-induced IFN-γ and IL-6, which are elevated in LPS-induced inflammation (Choi *et al*., 2013).

**2.5. Molecular Targets for Anti-Dengue Bioactive Compounds**

A normal consequence of most infections is an inflammatory response which includes transepithelial migration of immune system cells, such as dendritic cells, which is often mediated by factors which cause an increase in vascular permeability such as cytokines and matrix metalloproteases. MMPs are zinc- and calcium-dependent endopeptidases produced by cells which degrade the extracellular matrix surrounding cells resulting in reduced cell adhesion. MMPs have been implicated as important players in many biological processes as well as disease states, such as cancer (Roomi *et al.,* 2009). The family of MMPs comprises 22 different members so far (Somerville *et al*., 2003). Over 20 various MMPs have been identified that act on a broad range of substrates, including collagen type I, II, III, IV and stromyelin and are divided into subgroups based on their structure and substrate specificity (Nelson *et al*., 2000). These subgroups include gelatinases, stromelysins, collagenases, stromelysin-like, matrilysins and membrane-type MMPs (MT-MMPs). Fig. 2.6 depicts an overview of the domain organization of different MMPs**.** MMPs have several structural

features in common which include the “cysteine-switch” containing propeptide domain , hemopexin-like domain and the catalytic zinc-binding domain with the sequence HEXGHXXGXXHS. MMP-activity after secretion is regulated by the binding of TIMPs**.** TIMPs are a mammalian protein family composed of TIMP-1, -2, -3, and -4, which together display wide-ranging sequence homology and structural identity. TIMPs have been reported as natural MMP inhibitors that prevent the degradation of the ECM by abolishing the hydrolytic activity of all activated members of the metalloproteinase family, in particular that of membrane type 1-MMP (MT1-MMP), MMP-2, and MMP-9 (Jinga *et al*., 2006).

Control over MMP and/or TIMP activity *in vivo* occurs at different levels and involves factors such as regulation of gene expression, activation of zymogens and inhibition of active enzymes by specific inhibitors. Many MMPs and TIMPs are regulated at the level of transcription by a variety of growth factors e.g. cytokines and chemokines (Baker *et al*., 2002; Yan and Boyd, 2007).

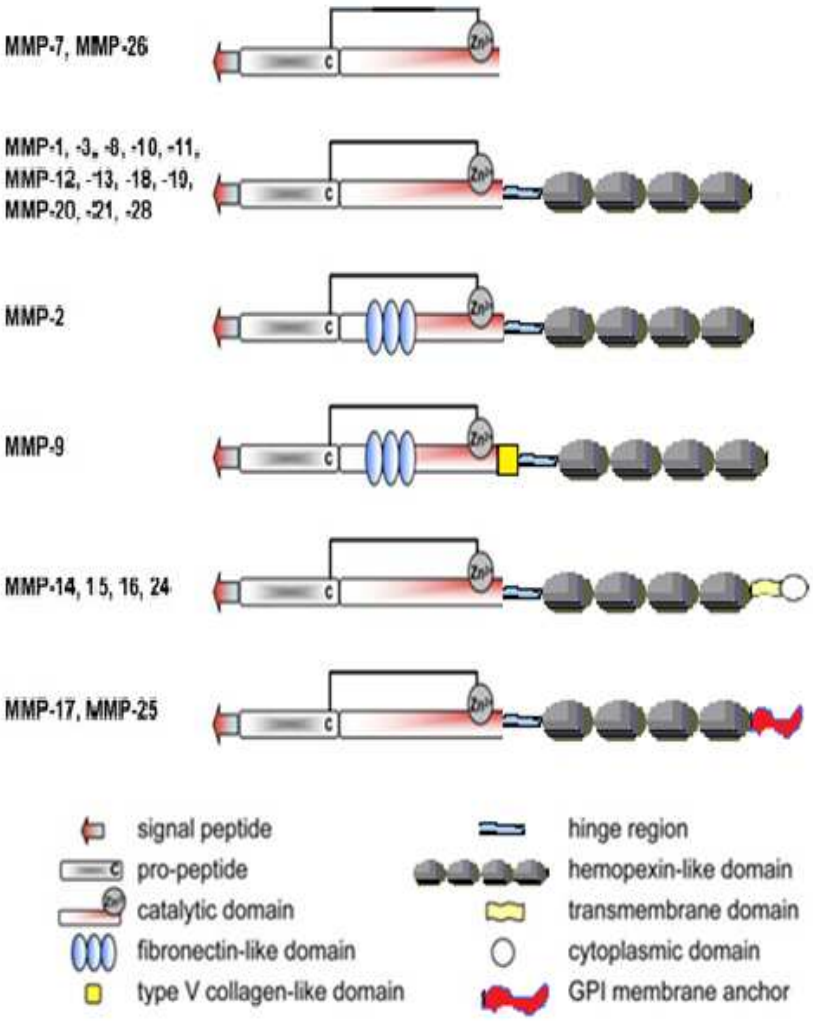



**Fig. 2.6 Domain organization of different mammalian MMPs.** GPI (glycosylphosphatidylinositol) membrane anchor. Figure modified from Hemmann *et al*., (2007).

Gelatinolytic MMPs (gelatinases), especially MMP-2 (gelatinase A) and MMP-9 (gelatinase B), play a key role in degradation of two main components of

ECM i.e. type IV collagen and gelatin. MMP-2 and MMP-9 are secreted in extracellular matrix in their latent zymogenic form, 72 and 92 kDa, respectively. MMP-2 and MMP-9 are cleaved by other MMPs or proteases to yield the activated forms of 68, 58 and 54 kDa for MMP-2, and 84 kDa for MMP-9. Many human tumors, like ovarian, breast and prostate tumors, and melanoma showed increased expression of MMP-2 and MMP-9 (Berube *et al*., 2005, Sato *et al*., 2004, and Di Nezza *et al*., 2002). Type IV collagen is a major structural protein for ECM and basement membrane.

MMPs role in the pathogenesis of severe dengue has been investigated. Luplertlop, *et al*. (2006) reported that immature dendritic cells overproduced MMP-9 and MMP-2 in lesser extent following DV infection in a virus dose dependent manner, In addition, conditioned cell culture media from dengue infected cells has been shown to have the ability to increase the permeability of an endothelial monolayer, both *in vitro* and *in vivo*, while inhibitors of MMPs and anti-MMP antibody were able to block the permeability induced by the media. These data strongly suggest that MMPs could be significant target in controlling the vascular permeability seen in dengue infection.

Similarly, infection of primary human MVEC results in overproduction of MMP-2 and to a lesser extent of MMP-9, leading to enhanced endothelial permeability. And the permeability was associated with loss of expression of the vascular endothelium-cadherin cell-cell adhesion (Luplertlop and Misse, 2008).

Epigallocatechin-3-gallate (EGCG) from green tea (*Camellia sinensis*) have been shown to have MMPs inhibitory activity (Fang et al., 2015).

**2.6. MMPs, TIMPs and Dengue Infection**

Secretion of MMP-2 and MMP-9 is induced by either activation of pro-MMPS or an increase in transcription of MMP gene which is regulated by various factors such as cytokines, growth factor and hormone or by cellular contacts acting through specific signalling pathways (Van den Steen *et al*., 2002).

Reports show that bradykinin and anaphylatoxins produced during DV infection also trigger the activation of gelatinolytic MMP secretion (Hsieh *et al*., 2004; DiScipio *et al*., 2006). Elevated levels inflammatory cytokines like IL-6, IL-8, TNF-α, , TGF-ß, vascular endothelial growth factor following DV infection are responsible for vascular damage and consequently vascular permeability in DHF/DSS (Nguyen *et al*., 2004; Juffrie *et al*., 2000; Fernandez-Mestre *et al*., 2004; Agarwal *et al*., 1999; Tseng *et al*., 2005). Most of them trigger the activation of gelatinolytic MMPs production (Luca *et al*., 1997; (Li *et al*., 2003). Thus, complex of pathways trigger overproduction of gelatinolytic MMP leading to the vascular damage in DHF/DSS (Luplertlop *et al*., 2006).

Similarly, the study of Kubelka *et al*., (2010) reported that metalloproteinases were over-produced during dengue fever. The data were in accord with earlier studies from Luplertlop and collaborators. Researchers

showed an increase in circulating MMP-9, MMP-12 and MMP-13 levels in plasma from dengue fever patients though MMP-2 levels did not present relevant changes in comparison to that of healthy individuals. Data showed that level of MMP-9 was found to be higher in severe than that of mild dengue fever patients. 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 (Weg *et al*., 2014). Waidab *et al*., (2008) reported that the mRNA expression level of MMP-9 was significantly elevated in children with DHF suggesting the roles of this mediator in the pathogenesis.

TIMP-1 and TIMP-2 production were enhanced in DV infection but their production didn't establish the fine balance between the MMPs and TIMPs consequently leading to microvascular leakage (Luplerdlop *et al*., 2006). DV-infection of hepatocytes (HepG2) increased the levels of MMP-2 while infected monocytes (U937) promoted MMP-9 production DV-infected HepG2 cells up-regulated the mRNA expression of MMP-2, whereas infected U937 cells enhanced the expression of MMP-9 mRNA. DV infection induced TIMP-1 expression in U937 cells. However, lower expression of TIMP-2 was observed in infected HepG2 cells (Pannatas *et al*., 2015).

Therefore, it is imperative to develop the therapeutic approaches for vascular permeability with specific inhibition of gelatinolytic MMPs and their

natural inhibitors, which might be beneficial in controlling the morbidity and mortality associated to vascular leakage-induced in severe dengue.

**2.7. Zymography**

The expression of MMPs can be analyzed with several techniques. A widely used technique is substrate zymography, which identifies MMPs by the degradation of their preferential substrate and by their molecular weight. Zymography is described as simple, sensitive, quantifiable, and functional assays to analyze MMPs in biological samples (Hawkes *et al*., 2010). All types of substrate zymography originate from gelatin zymography. The techniques are the same except that the substrate differs depending on the type of MMPs or TIMPs to be detected. In zymography, the proteins are separated by electrophoresis under denaturing [sodium dodecyl sulfate (SDS)], nonreducing conditions. The separation occurs in a polyacrylamide gel containing a specific substrate that is co-polymerized with the acrylamide (Fernandez-Resa *et al*., 1995). During electrophoresis, the SDS causes the MMPs to denature and become inactive. The activation of latent MMPs during zymography is believed to involve the "cysteine switch" because the dissociation of $Cys^{73}$ from the zinc molecule is caused by SDS. After electrophoresis, the gel is washed, which causes the exchange of the SDS with Triton X-100, after which the enzymes partially renature and recover their activity (Woessner, 1995). Additionally, the latent

MMPs are autoactivated without cleavage (Oliver et al., 1997). Subsequently, the gel is incubated in an appropriate activation buffer. During this incubation, the concentrated, renatured MMPs in the gel will digest the substrate (Hawkes*et al*., 2010). After incubation, the gel is stained with Coomassie Blue, and the MMPs are detected as clear bands against a blue background of undegraded substrate (Hawkes*et al*., 2010). The clear bands in the gel can be quantified by densitometry (Woessner, 1995). Zymography is based on the following principles: (i) during electrophoresis, gelatin is retained in the gel; (ii) MMP activity is reversibly inhibited by SDS during electrophoresis; and (iii) the SDS causes the separation of MMP-TIMP complexes during electrophoresis. This enables the detection of MMPs and TIMPs independently of one another (Hawkes*et al*., 2010). An additional advantage of zymography is that both the proenzymes and the active forms of MMPs can be distinguished on the basis of their molecular weight (Woessner, 1995)

*Gelatin zymography*

Gelatin zymography is mainly used for the detection of the gelatinases, MMP-2 and MMP-9. It is extremely sensitive because levels of 10 pg of MMP-2 can already be detected (Kleiner and Stetler-Stevenson, 1994). Since MMP-2 and MMP-9 are proteinases belonging to the gelatinase family, this procedure allows the specific detection of these MMPs by using gelatin as a substrate in the gel.

Proteins will migrate based on their size through electrophoresis, using SDS as a protein-denaturing agent. Gelatinase proteins that pass across the gel matrix will digest the gelatin substrate incorporated in the gel such that MMP-2 and MMP-9 will be detected at 72 and 92 kDa as clear zones against the dark background (Troeberg and Nagase, 2003). MMP-2 and MMP-9 activity were assayed by gelatin zymography in many studies (Luplertlop *et al*., 2006; Luplertlop and Misse, 2008; Yanti, 2011, Lee *et al*., 2008; Weng *et al*., 2010; Ling *et al*., 2010).

87 extracts of oriental medicinal herbs that are used as traditional cancer treatment in Korea, were screened for anti-MMP-9 activity. The hexane, chloroform fractions and water extracts showed weak inhibitory effect on MMP-9 activity. However, butanol fractions of *Magnolia officinalis* REHD. et WILS, *Magnolia obovata* THUEB, *Magnolia officinalis* REHD. et WILS. var. biloba REHD. et WILS, *Cinnamomum cassia* PRESL and *Euonymus alatus* (THUNB.) SIEB showed a strong MMP-9 inhibitory activity (Seo *et al*., 2005).

**2.8. Permeability Assay**

The endothelial cell lining of the internal vasculature defines semi-permeable barrier between the blood and the interstitial spaces of thebody. This barrier is composed of intracellular adherent, tight, and gap junction complexes, as well as desmosomes (Bingmei, 2001). Junction substructure components such

as connexins, integrins, cadherins, catenins, occludins, desmoplakins, selectins ,and PECAM-1 all act as interface regulators for paracellular permeability of nutrients, chemicals, and ions (Navarro *et al*., 1995; Dejana *et al*., 1996). Endothelial cell adhesive characteristics provide strength and stability for neighboring cells and the cellular cytoskeleton by interacting with actin and myosin contractile filaments Dudek *et al*., 2001). Junctional molecules also influence cell signaling and trigger responses that are translated into cell morphology changes and physiological angiogenesis (Krizbai *et al*., 2003).

A multitude of vasoactive cytokines, growth factors, and signal modulators react with endothelial cell substructure components to control permeability. VEGF, Interleukin-α and β, TNF-α, and IFN-γ have been shown to increase endothelial monolayer permeability (Lal *et al*., 2001; Burke-Gaffney *et al*., 1993; Marcus *et al*., 1996). Disruptions of the barrier integrity are manifested as microvascular hyperpermeability, which is associated with many systemic disease states. Increases in tissue permeability may be caused by weak, hemorrhaging vessels that become oedematous, and intensifies with irregular fluid flow through the vessels (Bates et al., 2002).

An essential ingredient of any *in vitro* permeability study is an intact, confluent cell monolayer. Endothelial cell monolayers cultured on semi-permeable membranes have been shown to form adherent and tight junctions (Esser *et al*., 1998). The *In Vitro* Vascular Permeability Assay provides an efficient

system for evaluating the effects of chemicals and drugs on endothelial cell permeability.

*Test Principle*

*In Vitro* Vascular Permeability Assay is ideal for measuring compounds that disrupt an endothelial monolayer. This Assay is performed in a 24-well tissue culture plate with 12 cell culture inserts. The inserts contain 0.4µm symmetrical pores within a transparent polyethylene membrane. The high pore density membranes permit amplified rates of basolateral diffusion with molecules of interest for permeability assays. The membrane is tissue culture treated on both sides for cell growth.

MDCK cells are seeded onto transwell inserts. The monolayer of MDCK cells forms in several days, which occludes the membrane pores. The cell monolayer is then treated with conditioned cell culture media collected from DENV cells, containing MMPs. After treatment, fluorescently labelled dextran (FITC-conjugated 70 kDa dextran) is added on top of the cells, allowing it to permeate through the cell monolayer. The extent of permeability is determined by measuring the fluorescence or optical density of the plate well solution below the monolayer (Chemicon International; *in vitro* Vascular Permeability Assay Kit with slight modifications).

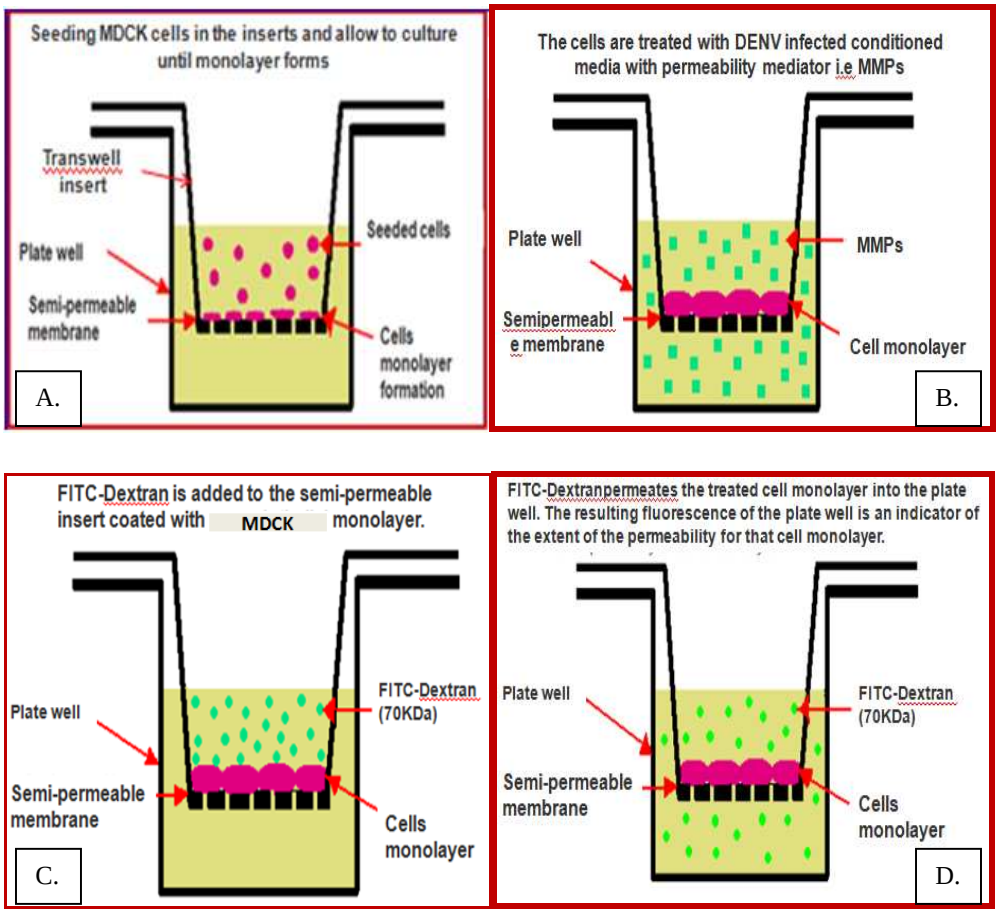



**Fig. 2.7 *In vitro* permeability assay.** MDCK cells are seeded in the transwell inserts containing 0.4µm symmetrical pores within a transparent polyethylene membrane and allowed to culture until the monolayer forms. The endothelial monolayer forms in several days, which occludes the membrane pores (A). Cell monolayers are treated with conditioned media collected from DV-infected cells containing permeability mediators like MMPs (B). FITC-Dextran is added to the semi-permeable insert coated with an endothelial monolayer (C). FITC-Dextran permeates the treated cell monolayer into the plate well (D). The resulting fluorescence of the plate well is an indicator of the extent of the permeability for that cell monolayer. Adapted from Chemicon International; *in vitro* Vascular Permeability Assay Kit with slight modifications.

# CHAPTER 3

# THE RESEARCH METHODS

This study demonstrated the effect of *Zingiber officinale* rhizome crude extracts and its biologically active component, pure [6]-gingerol, to facilitate protective host response during dengue infection by modulating the activities of MMP-2, MMP-9 and gene expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. Methods and procedures utilized in the study are illustrated in Figure 3.1.

## 3.1. Chemicals

Minimum Essential Medium (Cat. No. 11700-077), Fetal bovine serum (Cat.No. 10500-064), Penicillin-Streptomycin (Cat. No. 15070-063), L-glutamine (Cat. No. 25030-081), Non-essential amino acids (Cat. No. M7145), Sodium bicarbonate (Cat. No. 11810-033) were obtained from Invitrogen Life Technologies (USA). Dimethyl sulfoxide (Cat. No. D5879) and [3-(4, 5-dimethylthiazol-2-yl) - 2, 5-diphenyltetrazolium bromide] (Cat. No. M2128) were purchased from Sigma-Aldrich, Singapore. Qiagen RNA Isolation Kit and RT-PCR kit were purchased from Qiagen, Germany. Hexane, ethyl acetate, methanol, chloroform were purchased from Merck Chemicals Philippines. Corning/Costar, Gallic Acid 97.5-102.5% (titration) (Sigma G7384) and Gelatin, Type A, from

porcine skin, bloom 175 (Sigma Cat. G2625) were purchased from Chemline Scientific. Real time RT-PCR Master Mix were purchased from Life Technologies. 40% acrylamide / Bis solution 29:1 (Cat 170-6537), 1.5M Tris-HCl, pH 8.8 (Cat 161-0798), Ammonium persulfate (161-0700), TEMED and 0.5M Tris-HCl, pH 8.8 (161-0700) were purchased from Lifeline Diagnostic Supplies Inc. Bench Mark Protein Marker (Cat 10747-012) were purchased from MEDTEST, Medical Test Systems, Inc.

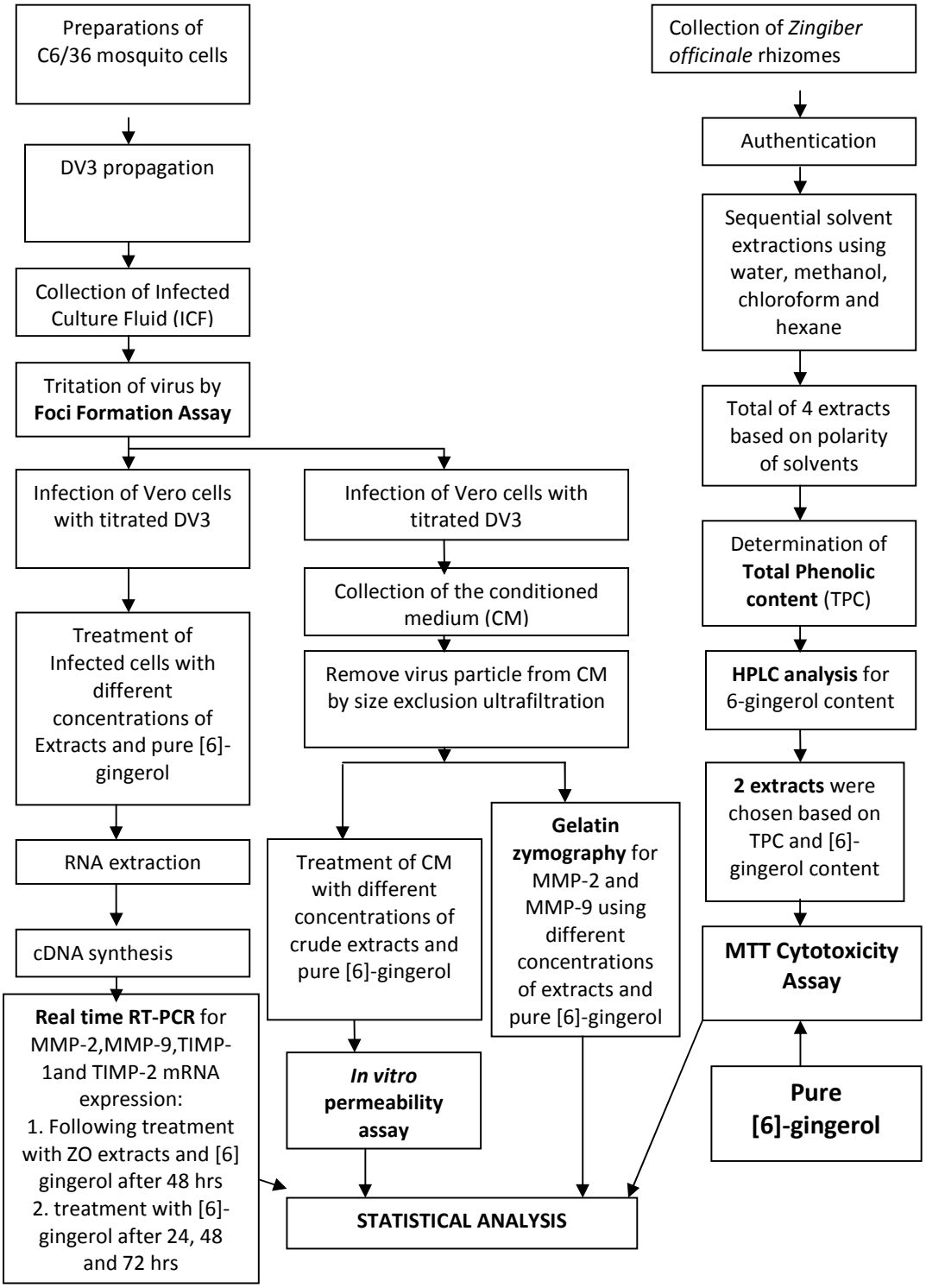



**Fig.3.1 General schematic diagram of the methods and procedures in the study.**

**Phase I. Plant Extract Preparation; Total Phenolic Content and 6-[gingerol] Content determination; Cell Culture; Dengue Virus Propagation; Foci Formation Assay; Cytotoxicity Assay:**

### 3.2. Collection and Preparation of *Zingiber officinale*rhizomes

About six kilograms of the fresh mature rhizomes (fibrous and nearly dry those harvested at nine months after planting) of *Zingiber officinale* were collected from the local supplier Klondykes Tuba, Benguet, Philippines. The freshly collected specimen was submitted to the National Museum for verification and issuance of a specimen voucher number (Appendix B). Vouchered specimens were processed at the laboratory of Research and Biotechnology Division of St. Luke's Medical Center, Quezon City, Philippines.

Taxonomically identified rhizomes of *Zingiber officinale* were washed with clean tap water to remove dirt and other particles. Excess water was drained prior to further washing with distilled water. The rhizomes were then peeled and cut into cross-sections of 2 ± 1 mm thickness followed by drying at $60^0$C, a temperature shown to produce significant amount of phenolics and antioxidant activity (Chumroenphat *et al*., 2011) and grinding until a fine powder was produced using a blender. The powder obtained was packed in polyethylene bags and then was stored in a refrigerator at 4 ° C for further uses.

### 3.3. Plant Extract Preparation

Plant extraction was adapted from optimized procedures of the antiviral study group of the Research and Biotechnology Division, St. Lukes Medical Center which was adapted and modified from Loh *et al*., (2009). Thirty grams (30g) of powdered plants was kept in a flask filled with 350 ml of water and was subjected to continuous shaking for five days. After five days, the plant-water suspension was centrifuged at 15000rpm for 20 minutes at $4^0$C. Supernatant was stored at $4^0$C to be lyophilized. The solid phase was placed into the cellulose thimble and connected to the soxhlet extraction apparatus for further extraction. Sequential extraction was done using 350 ml each of methanol ($80^0$C), chloroform ($70^0$), n-hexane ($80^0$C). For each extracting solvent, 16-hour cycles of extraction was performed. Two batches of 30g samples were extracted. The extracts in each solvent was collected and their volumes reduced using a rotary evaporator (Heidolph VV2000, Christ$^R$; Alpha 2-4 LSC) at 250 mm Hg at $63^0$C for methanol, 200 mm Hg at $55^0$C for Chloroform and 350 mm Hg at $60^0$C for n-hexane. Extracts using the same solvents from two batches were pooled together to avoid batch to batch variation. Aqueous and methanol extracts were lyophilized using a Christ LCG lyophilizer, (Lyo Chamber Guard; 121550 PMMA Burket; Germany).The chloroform and hexane extracts were dried using a SpeedVac Concentrator (Savant Automatic Environmental Speed Vac®with vapo Net®AE S2000). The weight of the extracts obtained of the plant material was measured. All extracts were stored at 4°C until use (figure 3.2).

A purified preparation of [6]-gingerol (> 98.0% pure) was purchased from Sigma Aldrich (Singapore). [6]-gingerol was dissolved in sterile DMSO at a stock concentration of 100mg/ml and stored at -20$^{o}$C. [6]-gingerol was diluted in cell culture medium for the bioassays such as cytotoxicity assays, permeability assays and gene expression assays. For zymography stock [6]-gingerol was diluted in incubation buffer to achieve the required working concentrations.

Total phenolic content (TPC) in extracts was determined by using Folin-Ciocalteu colorimetric methodology. All extracts were subjected to HPLC analysis for [6]-gingerol content. Extracts as indicated by quality and quantity of TPC and 6-gingerol were used for the *in vitro* cell-and enzyme-based assays. The extracts were prepared by dissolving in 0.1% dimethyl sulfoxide (DMSO) and filtered sterilized with 0.22µm syringe filters (Milipore) following dilution using Minimum Essential Medium (MEM) before treatment for the bioassays.

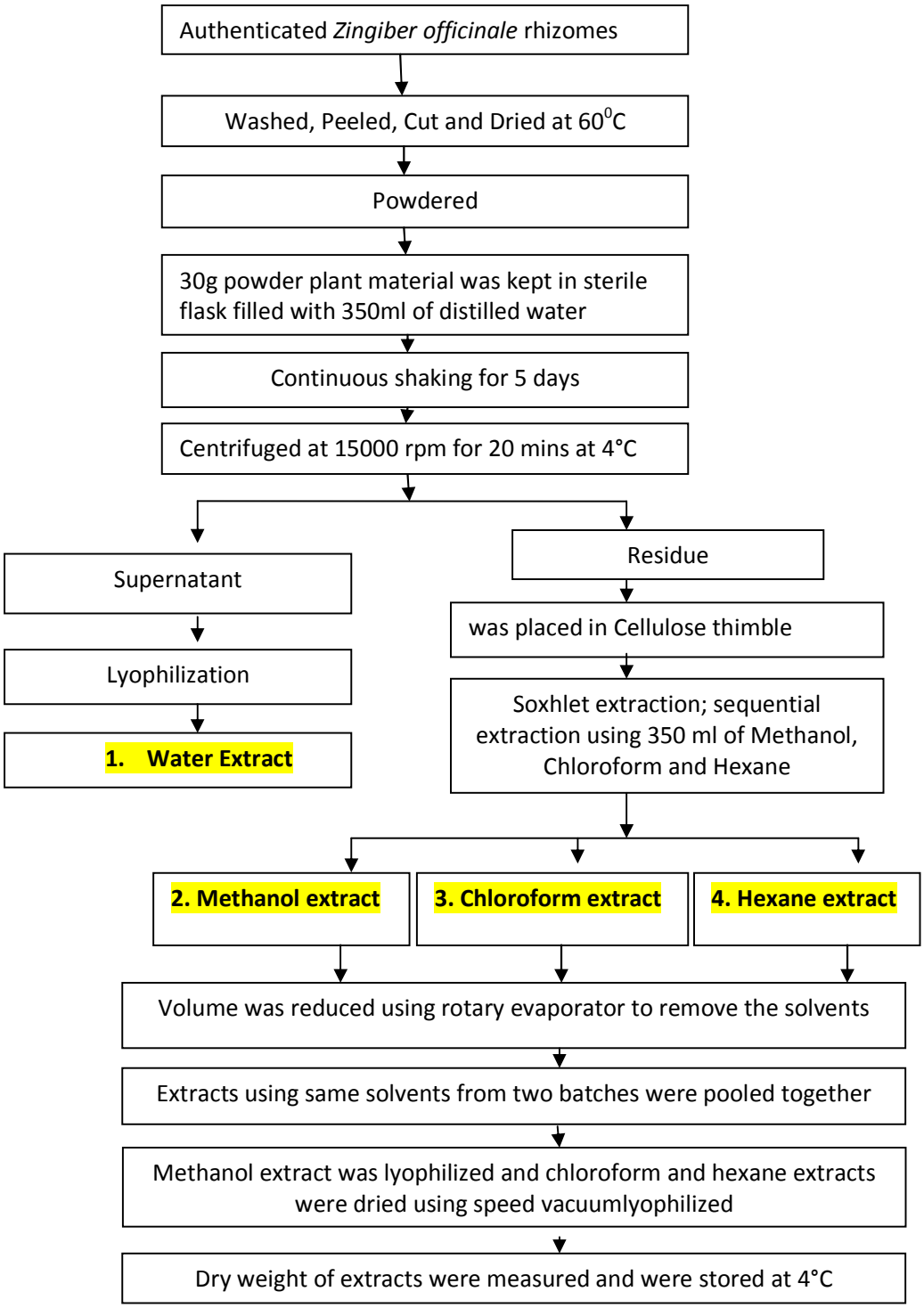



**Fig 3.2 Schematic diagram of solvent extraction from *Zingiber officinale* rhizomes.** (Adapted from Loh *et al*., 2009).

**3.4. Determination of Total Phenolic Content**

Total phenolic constituents of *Zingiber officinale* rhizome extracts was determined by utilizing the Folin–Ciocalteu reagent, in accordance to a method based on Loh *et al*. (2009) utilizing gallic acid as a standard. Weighed, *Z. officinale* rhizome aqueous extract (ZOA), methanol extract (ZOM), chloroform extract (ZOC) and hexane extract (ZOH) were reconstituted with their respective solvents to a concentration of 3 mg/mL. An aliquot of 20µL of each extract was added to 1.58mL of water in a cuvette followed by the addition of 100 µL of Folin–Ciocalteu reagent. After mixing thoroughly, the mixture was incubated at room temperature for 5 min. 300 µL of sodium carbonate solution (20%, w/v) was added and the mixture was incubated for 2 hours at room temperature. The absorbance of the mixture was measured at 765nm (Spectro UV/VIS Auto, LaboMed, inc, USA) against a blank. The total phenolic content was determined from the gallic acid calibration curve and was expressed as mg of gallic acid equivalents (GAE) per gram of extracts. All measurements were performed at least in triplicate, and presented as mean ± SD. Total phenol content was calculated using the following equation:

$C = c.V/m$

Where C is the total content of phenolic compounds in mg GAE/g of extract; c the concentration of gallic acid obtained from the calibration curve in mg/ml; V the volume of extract in ml and m is the weight of extract in grams.

### 3.5. HPLC Analysis

#### 3.5.1. Preparation of 6-gingerol Standards and Sample Extracts for HPLC

[6]-gingerol used for preparing the standards was purchased from Sigma–Aldrich (USA) (Cat. No. 29150-4). For HPLC [6]-gingerol standard was prepared by dissolving in HPLC-grade methanol to a stock concentration of 1mg/mL each and further diluted to a stock of 800 µg/ml. Serial dilutions of the 800.0µg/mL standard were made to produce the 400.0, 200.0, 100.0, 50.0 and 25.0µg/mL working standards. All six different concentrations of [6]-gingerol standards were capped and stored at 4 °C until used.

Lyophilized aqueous extract and dried methanol, chloroform and hexane extract of *Z. officinale* were dissolved in their respective solvents to a concentration of 3mg/mL. Prior to analysis, the standards and samples were filtered through a 0.22 µm syringe filter before injecting onto the HPLC system (Adapted from Loh *et al*., 2009).

#### 3.5.2. HPLC chromatographic analysis

The contents of 6-gingerol in the extracts was analyzed by HPLC system using Spectra HPLC system (TSP, USA) equipped with P2000 binary pump, AS1000 autosampler, UV2000 UV detector and SW4000 system controller. ZOM, ZOA, ZOC, ZOH (20µL) were subjected to HPLC for the 6-gingerol analysis. Separation was performed on a Lichrospher $R_{18}$ (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). [6]-gingerol in the extracts was identified and quantified based on retention time using [6]-gingerol as HPLC external standard which was purchased from Sigma–Aldrich (USA) (Cat. No.29150-4). A standard curve of the different concentrations of 6-gingerol at 25, 50,100, 200, 400 and 800µg/mL was prepared. The concentration of [6]-gingerol in the extracts were determined by linear regression. Method adapted from Puengphian and Sirichote (2008), with minor modifications.

**3.6. Cell Culture**

The different cell lines used in this study were obtained from the Research and Biotechnology division (RBD), St. Lukes Medical Center, Quezon City, Philippines which were purchased from American Tissue Culture Collection (ATTC, USA). Four cell lines were maintained for this experiment: C6/36 cells, Baby Hamster Kidney (BHK-21) cells, Vero cells and Madin-Darbin canine Kidney (MDCK) cells (Appendix F). C6/36 cell line is a cloned cell line derived from larvae of *Aedes albopictus* mosquito and used for dengue virus propagation while BHK-21 cell line is baby hamster kidney derived from *Mesocricetus auratus* and used for titration of propagated dengue virus. Vero cells are *Cercopithecus aethiops* (monkey) kidney cells and infected with dengue virus for MMP induction and

gene expression analysis. MDCK cells are epithelial cells derived from a kidney of an adult female cocker spaniel and were used for permeability assay in this study. All cell lines were propagated using conventional cell culture methods (Appendix F.5). Briefly, cells were maintained in Minimum Essential Medium (MEM, pH 7.2) supplemented with 20 mM L-glutamine, non-essential amino acids, 10% heat inactivated fetal bovine serum, 50U/mL penicillin, 50µg/mL streptomycin and $NaHCO_3$ (all from GIBCO Invitrogen Life Technologies, USA). Spent media was removed by decanting. Adherent cells were washed with PBS to remove debris and dead cells. After washing, cells were trypsinized for five minutes at 37°C to detach the cells. The reaction was terminated by addition of growth media. Collected cells were centrifuged at 1000 rpm for three minutes to obtain a cell pellet. The supernatant was removed by decanting and the cell pellet was washed with PBS to remove debris, dead cells, and traces of trypsin. The supernatant was discarded and the cells were resuspended with 5 ml growth medium. Cell count and viability was estimated using Trypan blue dye exclusion. 95% to 100% viability of cells was used for plating. Vero cells were seeded at 2.5 x $10^5$ cells/well on a 12-well plate. Plates and T-25 flasks were incubated at 37°C with 5% $CO_2$ for 3 days until confluent for BHK-21, Vero and MDCK cells while C6/36 cells were incubated at 28°C without $CO_2$.

**3.7. Propagation of Dengue Virus in *Aedes albopictus* C6/36 Cells**

Clinical isolates of dengue virus-3 (DV3)were propagated in monolayers of C6/36 cells cultured in 25cm$^2$ tissue culture flasks using Leibovitz-15 (pH 7.2) media supplemented with 2% heat inactivated fetal bovine serum, non-essential amino acids, 20mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from GIBCO Invitrogen Life Technologies, USA) for 14 days at 28$^0$C in the absence of $CO_2$. Infected culture fluid (ICF) was collected on days 7 and 14 days post-infection [procedure adapted from Jain *et al*., (2008) with minor modifications]. The propagated virus was stored at -80°C in aliquots until use in infection studies.

**3.8. Viral Infectivity Titration by Focus Formation Assay (FFA)**

Cell culture supernatants from infected mosquito C6/36 cells were assayed for infectious DV3 by focus formation assay. The titer of infectious particles was quantified and reported as foci-forming units per mL (ffu/mL). Immunoperoxidase staining was performed to detect and quantitate dengue virus infectivity titer in BHK-21 [C-13] cells. DV3 was titrated by foci formation assay as described previously by Zandi, (2011) with slight modification. Briefly, a BHK-21 [C-13] cell monolayer was prepared in 96 well cell culture microplates. After attaining ~80% confluency, growth medium was removed and the cells were infected by DV3 (100 µL of serially diluted infected culture fluid) for 2 hours

to allow virus attachment and penetration into the host cell. Thereafter, wells were overlaid with 2% carboxymethylcellulose in Eagle's Minimum Essential Medium (2% CMC-EMEM) and incubated for 48 hours 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. 5% formaldehyde (JK Baker, USA) was added to fix the cells for 2 hours at room temperature (RT) followed by three washes with PBS. Fixed cells were permeabilized using 50 µL of 1% Nonidet P-40 (Sigma-Aldrich, Singapore) for 1 hour. Cells were washed three times with PBS and blocked using 50 µL of BlockAce$^{TM}$ (Wako, Japan) for 1 hour. Following blocking of nonspecific sites and another three washes 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 presence of viral antigens. The reaction was allowed to take place for 1 hour at RT. Cells were washed three times with PBS and incubated with goat anti-rabbit IgG conjugated with horse-radish peroxidase (HRP) at final dilution of 1:100 (Calbiochem, USA) as a secondary antibody for 1 hour at RT. Finally, fifty microliters 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 minutes. When color development was observed, 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). The virus titer of each ICF with countable foci in BHK-21 cells was computed using the following formula: *FFU/ml = [(Ave. number of foci) x (dilution factor at which foci were counted)] / volume of inoculum in ml.*

**3.9. Cell-and Enzyme-Based Assay**

**3.9.1. 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay**

Cytotoxic effects of the test extracts and pure [6]-gingerol on cultured cells was measured using MTT assay according to the method described previously with slight modicications (Denizot and Lang, 1986), which is based on the conversion of MTT to formazan by mitochondrial enzyme. Briefly, monolayers of Vero cells were seeded in 96-well microplates and treated with different concentrations of extracts and pure [6]-gingerol in triplicate. Test extracts were filter sterilized prior to treatment using 0.22µm syringe filter. 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 (eg. DMSO) to each well. The optical density of the wells was measured at 570 using a 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 extracts and pure [6]-gingerol for treatment were adjusted based on the $IC_{50}$.Bleomycin Sulfate was used as positive control for cell toxicity. Negative control was cells treated with media alone (without any inhibitors).

**Phase II. Cell-and Enzyme-based Assay: Analysis of the modulatory activity of *Zingiber officinale* rhizome crude extracts and pure [6]-gingerol on the activity and mRNA expression of MMP-2, MMP-9, TIMP-1, TIMP-2 in DV3 infected Vero cells; *in vitro* permeability assay:**

### 3.9.2. Determination of MMPs Activity by Gelatin Zymography Assay

#### 3.9.2.1. Experimental setting for MMP Induction

*Infection of Vero cells with DV3 and collection of the conditioned media*

Infection of cells with DV3 and collection of conditioned media (CM) was performed according to a method described previously by Luplertlop and Misse (2008) with slight modifications. Briefly, Vero cells were seeded at a density of 2.5 x $10^5$ cells/mL in T-25 flasks and cultured for 24 hrs at $37^0$C with 5% $CO_2$ using MEM with 10% FBS (until 80-90% confluency is reached). The monolayer of cells was washed with 1X PBS and adapted to serum free MEM for 24 hours. The adapted cell monolayer was exposed to DV3 (moi of 1 ffu/cell) for 4 hours to allow the virus to adsorb with gently shaking of flasks every 30 minutes, washed

twice to remove unadsorbed virus, and further cultured at $37^0$C with 5% $CO_2$ for 24 hours in MEM, without FBS.

The conditioned serum-free cell culture media from dengue-infected Vero cells was collected in sterile 15mL conical tubes. The removal of virus particles was done by using size exclusion ultrafiltration using Amicon$^{TM}$-4 Centrifugal Filter Units (Millipore) with molecular cut off 100kDa and MMPs were concentrated by centrifugation using Amicon$^{TM}$-4 Centrifugal Filter Units (Millipore) with molecular cut off 30kDa. Thus collected, concentrated conditioned media were aliquoted in 1.5 ml micro-centrifuge tubes and were stored at -$20^0$C until use.

All further bioassays were performed using virus-free (negative reverse transcription–PCR according to Deubel *et al*., 1990) and concentrated CM containing the soluble factors produced in 24 hours after the viral washing out, that is, 4 h after infection.

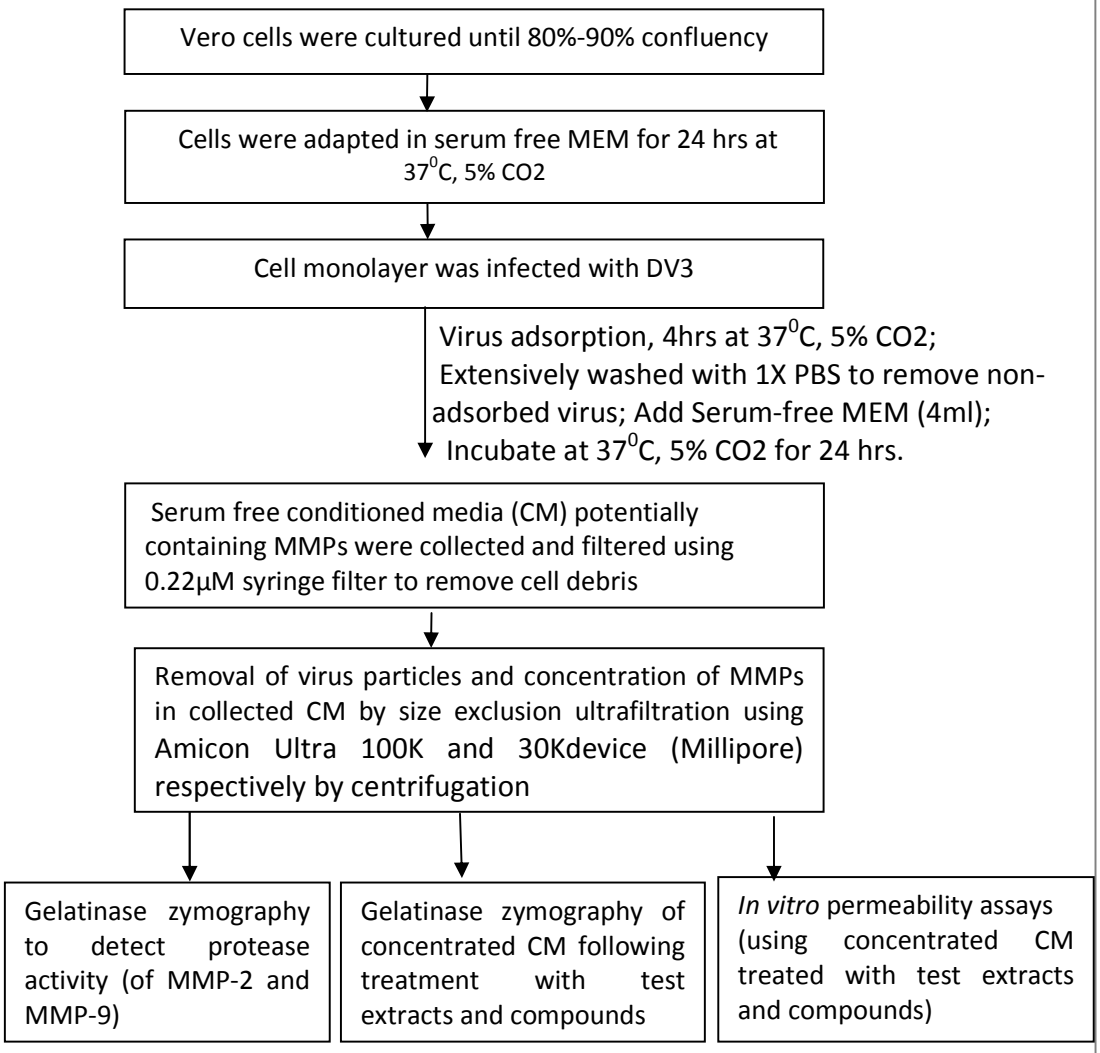



**Fig 3.3 General Schematic diagram of experimental design for zymography and permeability assay.** (Adapted from Luplertlop and Misse, 2008 with minor modifications)

**3.9.2.2. PCR detection for absence of viral genome from collected conditioned media (CM)**

Reverse transcription–polymerase chain reaction (RT-PCR) and agarose gel electrophoresis (AGE) was performed to ensure the absence of dengue viral

genome in the collected conditioned media collected from DV3 infected Vero cells after filtration using 100kDa Amicon ultra centrifugal units. All bioassays were performed using conditioned media collected from DV3 infected Vero cells that is free of virus particles and contained the soluble factors (eg. MMPs) produced in 24 h after the viral washing out, that is, 4 h after infection. Dengue virus RNA was extracted using QIAamp® Viral RNA Mini Kit (Qiagen, Germany) following manufacturer's instructions. cDNA synthesis was carried out using Superscript III Reverse Transcription Kit (Invitrogen, USA) according to Lanciotti et al. (1992) using the following reaction components:

| Reagent | Volume per Reaction (µL) | Concentration |
|---|---|---|
| 5x Reverse Transcriptase buffer | 4.0 | 1x |
| 200 U/µL Reverse transcriptase | 0.5 | 5 U/µl |
| 20 U/µL RNAse inhibitor | 0.5 | 0.5 U/µl |
| 0.1 M DTT | 2.0 | 10 µM |
| 2.5 nM dNTP | 1.5 | 0.18 nM |
| 50 µM Random primers | 0.5 | 1.25 µM |
| Nuclease-free water | 1.0 | - |
| RNA template | 10.0 | - |
| Total | 20.0 | - |

The reaction was incubated at 37°C for 1 hour. Thereafter, the presence of the DV genome was detected using primers: forward 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' and reverse 5'-TTGCACCAACAGTCA ATGTCTTCAGGTTC-3' (Lanciotti *et al.*, 1992) that spans the core to pre-membrane region. The following is the PCR reaction set-up and the corresponding cycling conditions:

| Reagent | Volume per Reaction (µL) | Concentration |
|---|---|---|
| 2x Premix Taq (Vivantis) | 12.5 | 1x |
| 25 µM Forward primer | 0.5 | 500 nM |
| 25 µM Reverse primer | 0.5 | 500 nM |
| Nuclease-free water | 9.0 | - |
| cDNA template | 2.5 | - |
| Total | 25.0 | - |

| | | | |
|---|---|---|---|
| Initial denaturation | 94°C | 3 minutes | |
| Denaturation | 94°C | 30 seconds | 30 cycles |
| Annealing | 56°C | 45 seconds | |
| Elongation | 72°C | 1 minute | |
| Final elongation | 72°C | 5 minutes | |

**3.9.2.3 Gelatin Zymography Assay**

The activity of MMP in the conditioned medium was determined by gelatin zymography protease assays as described previously by Misse *et al*., 2001; Seo *et al*., 2005). Briefly, CM containing equal amounts of protein were mixed with nonreducing sample buffer followed by electrophoresis in 8% acrylamide gel containing 1mg/ml gelatin in the presence of sodium dodecyl sulphate (SDS). Following electrophoresis, the gels were soaked in 2.5% Triton X-100 (3 × 20 min) with gentle agitation to remove the SDS followed by rinsing in distilled water. For gelatinase inhibition assays, ZOA, ZOM and Epigallocatechin-3-gallate (EGCG) were freshly solubilized using the developing buffer which is used for developing the zymogram. Gel slab was then cut into the slices

corresponding to the lanes which were put in different tanks and incubated in the 10mL 2X developing buffer, containing 6.67 mL of 1.5 M Tris-HCl buffer (pH 8.8), and 0.4 g CaCl2• 2H2O and 0.02% Brij-58 filled to 100 mL with distilled $H_2O$ with or without 6.25µg/ml, 12.5µg/mL, 25µg/ml , 50µg/mL of the ZOM and 100 µM of EGCG for 20 hoursat 37 °C to allow digestion of the gelatin. After incubation, gelatinase 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 were determined by comparison with molecular weight protein 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). Gelatinase proteins that pass across the gel matrix digest the gelatin substrate incorporated in the gel such that MMP-2 and MMP-9 will be detected at 67kDa and 92 kDa as clear zones against the dark background (Troeberg and Nagase, 2003). The assessment of inhibitory activity was based on the measurement of content of clear zone, gelatinolysis of MMP-2 and MMP-9. Epigallocatechin gallate (EGCG) was tested for protease inhibitory activity as a control as EGCG has been shown to have a strong inhibition of the gelatinolytic activities of MMP-2 and MMP-9 (Fang *et al*., 2015). In order to determine the type of gelatinase observed on the zymograms, 10mM ethlenediaminetetraacetic acid (EDTA; Ca2+

chelator) was added to the buffer during the incubation period. All procedures were performed as they were for gels without EDTA in the incubation buffer.

### 3.9.3. Real-time PCR for MMPs and TIMPs Expressions

Quantitative Real-Time PCR was employed to measure changes in MMP-2, MMP-9, TIMP-1 and TIMP-2 expression following DV3 infection and treatment with test extracts and compounds for 48 hours at 37°C. Similarly expressions of MMP-2, MMP-9, TIMP-1 and TIMP-2 following DV3 infection and treatment with [6]-gingerol for 24 hrs, 48 hrs and 72 hrs were also measured. Final concentrations of extracts and pure [6]-gingerol were prepared in agreement with the pre-determined cytotoxicity data. Wells without the extract were mock-inoculated with media alone and serve as control. The positive control group was treated with EGCG.

#### 3.9.3.1 RNA Extraction

Vero cells were seeded at a density of 5 x $10^4$ cells/mL in a 24 well tissue culture plate and infected with DV3. After infection and treatment, the cells were harvested by trypsinization and centrifugation at 1000 rpm for 3 minutes and total RNA was isolated using RNeasy™ mini kit (Qiagen, USA) according to the manufacturer's protocol. Briefly, harvested pelleted cells were washed twice with 1x PBS (-) and suspended in RLT buffer for lysing the cells. After vortexing,

the lysate was pipetted directly into a QIAshredder spin column which was placed in a 2 ml collection tube, and was centrifuged for 2 min at full speed to homogenize the lysate. 1 volume of 70% ethanol was added to the homogenized lysate, and mixed by pipetting. The sample was transferred to an RNeasy spin column placed in a 2ml collecting tube and was centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. One wash step with buffer RW1 and two wash steps with RPE were followed. Finally RNA was eluted in RNase-free water and stored at -80$^0$C until use (Appendix M.1). The RNA was quantified using Nanodrop at 260nm, and its purity was assessed by determining the ratio of 1.9-2.1 A260nm/A280nm. (Based on RNeasy® Mini Handbook, September 2010)

**3.9.3.2 cDNA Preparation and Real Time RT-PCR**

Total RNA isolated from each cell pellet was reversed transcribed using iScript transcription supermix (Biorad, USA) in a 20 µl reaction volume. The concentrations of the components are shown below.

| Reagent | Volume per Reaction (µL) | Concentration |
|---|---|---|
| iScript transcription supermix | 4.0 | 1x |
| RNA template | var | 1µg |
| Nuclease-free water | var | |
| Total | 20.0 | |

The reaction was incubated as follows:

Priming: 5 minutes at $25^0$C
Reverse Transcription: 30 minutes at $42^0$C
RT inactivation: 5 minutes at $85^0$C

The resulting cDNA was stored at -$20^0$C and used as template in the subsequent PCR reaction.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 (Sen *et al*., 2009). Real-time PCR amplification was perfomed using iQ™ SSo FAST® Eva-Green Supermix (Molecular Probes BioRad, USA) in the presence of specific primer pairs. The following oligonucleotides were used as primers:

**Table 3.1 Primers for MMPs, TIMPs and GAPDH**

| Genes | Forward Primer 5'-3' | Reverse Primer 5'-3' | References |
|---|---|---|---|
| MMP-2 | AGGATCATTGGCTACACACC | AGCTGTCATAGGATGTGCCC | Luplerdlop*et al*., (2006) |
| MMP-9 | CGCAGACATCGTCATCCAGT | GGATTGGCCTTGGAAGATGA | Luplerdlop*et al*., (2006) |
| TIMP-1 | GCAACTCCGGACCTTGTCATC | AGCGTAGGTCTTGGTGAAGC | Ottino *et al*.,(2002) |
| TIMP-2 | GTAGTGATCAGGGCCAAAG | TTCTCTGTGACCCAGTCCAT | Ottino *et al*.,(2002) |
| GAPDH | CCACCCATGGCAAATTCCATGGCA | TCTAGACGGCAGGTCAGGTCCACC | Luplerdlop*et al*., (2006) |

PCR reactions were performed in 20 µL volumes. The components of the reaction and their respective concentrations are listed below. Real-time RT-PCR

was performed in Rotor Gene RG-3000 Real-Time PCR Detection System; using the thermocycler programs as follows:

The PCR was carried out at 95°C for 2 min followed by 45 cycles for all genes. Each cycle consisted of a denaturation step (at 94°C for 30s), an annealing step for 45s (at 56°C for MMP-2 and GAPDH, 59°C for MMP-9, $60^0$C for TIMP-1 and TIMP-2) and extension step (at 72°C for 1 min), followed by a final elongation step (at 72°C for 1 min). The melting temperature range is 55-90°C. To control variation in mRNA concentration, all results were normalized to the housekeeping gene, GAPDH.

| Reagent | Volume per Reaction (µL) | Concentration |
|---|---|---|
| iQ™ Eva® Green Supermix | 10 | 1x |
| 10 µM Forward primer | 1 | 500 Nm |
| 10 µM Reverse primer | 1 | 500 nM |
| Nuclease-free water | 6 | - |
| cDNA template | 2 | - |
| Total | 20.0 | - |

A real-time quantitative RT-PCR using relative quantitation by the comparative CT method was used to determine mRNA expression. Threshold cycle number (CT), of three independent reactions, was determined using the Rotor Gene RG-3000 Real-Time PCR Detection System and the mean CT was determined. The levels of specific gene expression were normalized to GAPDH levels using the formula $2-\Delta\Delta CT$, where $\Delta\Delta CT = \Delta CT$ (sample) − $\Delta CT$ (calibrator) and $\Delta CT$ is the CT of the housekeeping gene (GAPDH) subtracted from the CT of

the target genes. The samples are the Noninfected Vero cells, EGCG (100 µM and ZOA, ZOM and [6]-gingerol treated (50, 25, 12.5 and 6.25µg/ml) treated DV infected Vero cells for 48 hrs of incubation. The specificity of the products was also confirmed by melt curve analysis.

### 3.9.4 *In vitro* Permeability Assay

To demonstrate the preventive role of *Zingiber officinale* rhizome crude extracts (ZOA, ZOM) and pure [6]-gingerol on cellular permeability, *in vitro* permeability assay was performed using a 24-well tissue culture plate with cell culture transwell inserts according to manufacturer's instructions (Chemicon International; *in vitro* Vascular Permeability Assay Kit with slight modifications).

Briefly, MDCK cells were cultured onto transwell inserts (0.4 µm-pore, 6.5 mm-diameter Transwell; Costar) at the density of $2.5x10^5$ cells/ml to form confluent monolayer. These cells differentiate into columnar epithelium and form tight junctions when cultured on semi-permeable membranes. The monolayer formed occludes the membrane pores. To demonstrate if ZOA, ZOM and [6]-gingerol prevent the permeability, confluent monolayer of MDCK grown on 24-well transwell polyethylene membranes were exposed to the concentrated virus free CM collected from non-infected and DV-infected Vero cells (CM with MMP-2 and MMP-9 activities as demonstrated by zymography) for 24 hrs at $37^0$C in the presence or absence of various concentration of ZOA,

ZOM and [6]-gingerol and 100µM EGCG (Known gelatinolytic MMP inhibitor) (500 mL in the lower chamber and 350 mL in the upper chamber). After 24 hrs of incubation, all the treated spent media were removed and transwells were shifted to new well of the same plate followed by addition of fluorescein isothiocyanate–dextran (FITC-dextran-70kDa) to the top chamber. After 5 minutes of incubation, the chambers were removed and fluorescence was read directly in the lower chamber using a Perkin Elmer$^{R}$ LS55 Luminescence Spectrofluorometer with excitation at 485 nm and emission at 535 nm. Three independent and duplicate experiments were performed for the assay. Mean fluorescence intensity of control wells (concentrated CM from DV infected cells) was calculated and referred to as 100% permeability. Experimental controls included the use of known proteases, such as trypsin, to demonstrate that the proposed mechanism of permeability and the *in vitro* assay were valid. Values obtained with CM from DV noninfected, infected and different treatment group were then expressed as a percentage of control (Luplertlop *et al*., 2006; Luplertlop and Misse, 2008).

**3.10. Statistical Analysis**

Cytotoxicity tests and total phenolic content determinations were done in triplicate. HPLC experiments were done in three independent experiments with triplicate analyses while three independent experiments were performed

for zymography assay and Real time RT-PCR assay while three independent with duplicate analyses were performed for Permeability assay. Statistical analyses were performed using Graph Pad Prism for Windows, version 6 (Graph Pad Software Inc., San Diego, CA, USA).All data i.e. cell viability (cytotoxicity assay), MMP activity (gelain zymography assay), mRNA expression analysis (Real-time PCR assay) and cellular permeability assay were presented as the mean ± Standard Deviation (SD) and means compared using ANOVA followed by Tukey post hoc/Dunnet Multiple comparison test for multiple comparisons. For all measurement, differences will be considered statistically significant at $p<0.05$.

# Chapter 4

# RESULTS AND DISCUSSION

## 4.1 Preparation of Plant Extracts and Percentage Yield of *Zingiber officinale* rhizomes Aqueous (ZOA), Methanol (ZOM), Chloroform (ZOC) and Hexane (ZOH) extracts

The freshly collected mature rhizomes (6kg) was vouchered and issued with Control No: 694; O.R. No. : 1640084 (Appendix B) at the National Museum after verification. After sequential solvent extractions, the aqueous extract had highest retrieved dry weights of 4.790 grams followed by chloroform extract with 2.038 grams, methanol extract with 1.706 grams and hexane extract with 0.410 grams (Table 4.5). In terms of percentage (%) yield, aqueous had highest percent yield of 7.98% followed by chloroform, methanol and hexane extracts with 3.39%, 2.84% and 0.68%, respectively.

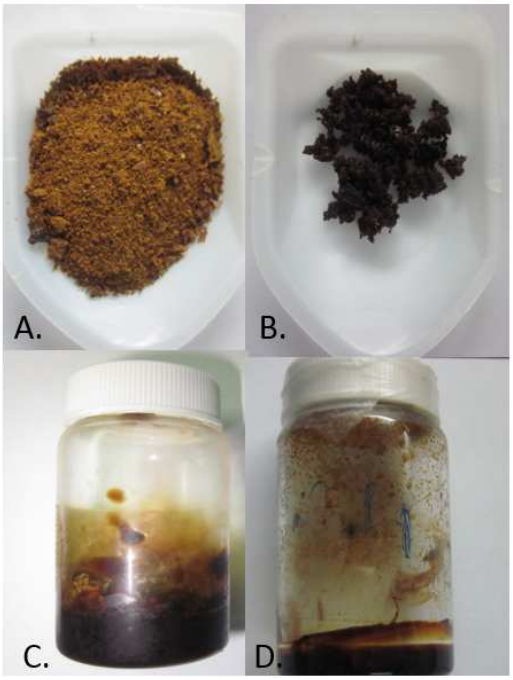



**Fig 4.1 Photomicrograph of *Z. officinale* rhizome extracts**. (A) *Z. officinale* rhizome aqueous extract (ZOA); (B) *Z. officinale* rhizome methanol extract (ZOM) (C) *Z. officinale* rhizome chloroform extract (ZOC) and (D) *Z. officinale* rhizome hexane extract (ZOH).

**4.2 Total Phenolic Contents of the Extracts**

Highly significant quantities of phenolic content were found in ZOM in terms of equivalents of gallic acid as compared to ZOA followed by ZOC and ZOH (Table 4.5). Equivalents of standards were calculated on the basis of standard regression lines for gallic acid ($R^2$ = 0.9992) (figure 4.2). Amount of total phenolics in terms of milligram gallic acid equivalents per gram (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 47.15±0.42, respectively (Table 4.5).

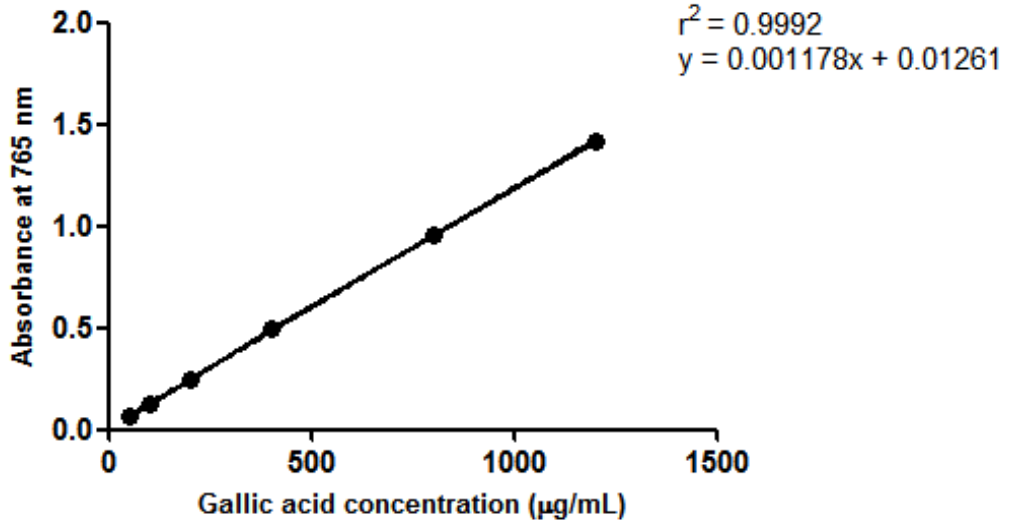



**Fig 4.2 Regression line with gallic acid with Folin-Ciocalteu reagent**

### 4.3 High performance liquid chromatography analysis

#### 4.3.1 Validation of HPLC method for the quantification of [6]-gingerol (Method Evaluation)

Before analysis of plant extracts, the HPLC method was validated. The repeatability and linearity were evaluated.

##### 4.3.1.1 Repeatability

***Intra-batch Repeatability***

Intra-batch repeatability was evaluated by repeated injections of [6]-gingerol standard solutions within the same day at short intervals of time. The

summary of intra-batch repeatability for six different concentrations of [6]-gingerol are given in Tables 4.1 and 4.2. For the retention time of [6]-gingerol, Coefficient of Variance (CV) values ranged from 0.1 to 0.9% while CV values ranged from 1.0 to 8.2% for the peak area of [6]-gingerol. Results obtained were repeatable since values of CV for both retention times and peak areas were all below 15% (Rivera *et al*., 2007).

**Table 4.1 Summary of intra-batch repeatability of [6]-gingerol standards using retention times.**

| Concentration (µg/ml) | Retention Time (min) | | | Mean RT | S.D. | CV (%) |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | | | |
| 25 | 6.81 | 6.70 | 6.71 | 6.74 | 0.05 | 0.87 |
| 50 | 6.78 | 6.72 | 6.71 | 6.74 | 0.03 | 0.57 |
| 100 | 6.71 | 6.71 | 6.7 | 6.71 | 0.01 | 0.06 |
| 200 | 6.69 | 6.67 | 6.69 | 6.68 | 0.01 | 0.16 |
| 400 | 6.69 | 6.66 | 6.69 | 6.67 | 0.01 | 0.27 |
| 800 | 6.67 | 6.65 | 6.67 | 6.66 | 0.01 | 0.15 |

**Table 4.2 Summary of intra-batch repeatability of [6]-gingerol standards using peak areas.**

| Conc. (ppm) | Peak Area | | | Mean Peak Area | S.D | CV (%) |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | | | |
| 25 | 308490 | 262717 | 288445 | 286550.7 | 22945.22 | 8.00 |
| 50 | 647888 | 658405 | 646104 | 650799 | 6647.11 | 1.02 |
| 100 | 1439722 | 1229514 | 1394094 | 1354443 | 110571.20 | 8.16 |
| 200 | 2984838 | 2766363 | 2904417 | 2885206 | 110497.20 | 3.82 |
| 400 | 6670612 | 6042370 | 6730612 | 6481198 | 381218.50 | 5.88 |
| 800 | 14748746 | 13999119 | 14159903 | 14302589 | 394657.70 | 2.75 |

***Inter-batch Repeatability***

The inter-batch repeatability was determined using the same method on identical test material, in one laboratory, wherein the operator and time were changed. For this study, [6]-gingerol standard solutions were compared on 3 different days.

Table 4.3 and 4.4 summarizes the retention times and peak areas of [6]-gingerol from three different trials on 3 different days. All CV values were below 15% giving acceptable results, thus, the method was repeatable.

**Table 4.3 Summary of inter-batch repeatability of [6]-gingerol standards using retention times.**

| Concentration (µg/ml) | Retention Time (min) | | | Mean RT | S.D | CV (%) |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | | | |
| 25 | 6.62 | 6.72 | 6.71 | 6.72 | 0.06 | 0.82 |
| 50 | 6.71 | 6.73 | 6.77 | 6.74 | 0.03 | 0.45 |
| 100 | 6.85 | 6.82 | 6.94 | 6.87 | 0.06 | 0.91 |
| 200 | 6.81 | 6.96 | 6.98 | 6.92 | 0.09 | 1.34 |
| 400 | 6.88 | 7.03 | 7.15 | 7.02 | 0.14 | 1.93 |
| 800 | 6.89 | 7.12 | 7.21 | 7.32 | 0.17 | 2.25 |

**Table 4.4 Summary of inter-batch repeatability of [6]-gingerol standards using peak areas.**

| Conc. (µg/ml) | Peak Area | | | Mean Peak Area | S.D | CV (%) |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | | | |
| 25 | 318510 | 321727 | 268555 | 302930.67 | 29813.62 | 9.84 |
| 50 | 745686 | 687216 | 755256 | 729386.00 | 36832.43 | 5.05 |
| 100 | 1343534 | 1514536 | 1353624 | 1403898.00 | 95948.04 | 6.83 |
| 200 | 3152758 | 3458157 | 2993789 | 3201568.00 | 236000.47 | 7.37 |
| 400 | 6857368 | 7136844 | 6568345 | 6854185.67 | 28462.86 | 4.15 |
| 800 | 15375937 | 16357934 | 18576329 | 16770066.67 | 1639517.38 | 9.78 |

**4.3.1.2 Linearity**

The linearity of an analytical method is the ability to yield results that are directly proportional to the concentration of analyte within a given range. Figure 4.3 shows a representative HPLC chromatogram at different concentration of [6]-gingerol. The calibration curves using peak area of [6]-gingerol at different concentration ranges, showed R-squared value of 0.9975 (figure 4.3).

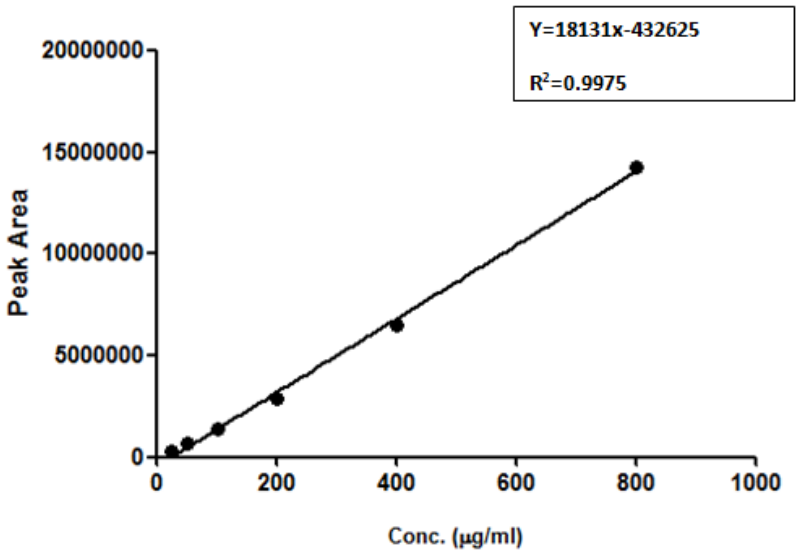



**Fig 4.3 The typical calibration curve of 6-gingerol standards determined by HPLC assay.**

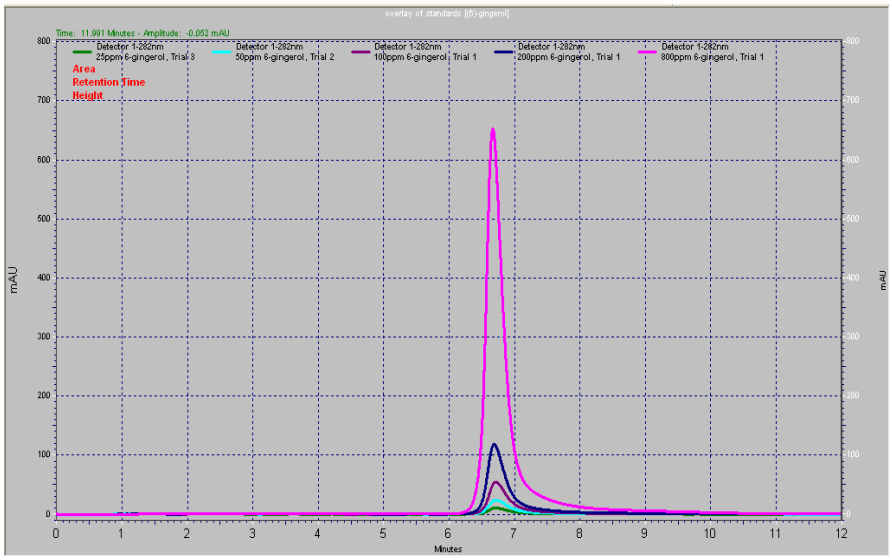


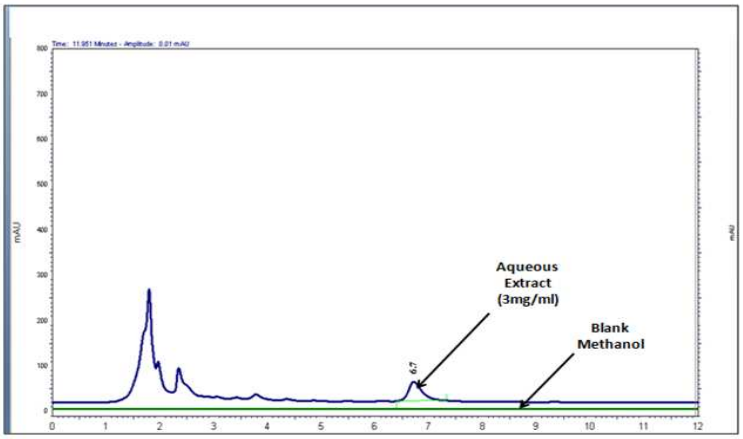

**Fig 4.4 The overlay of HPLC chromatograms of different concentrations of [6]-gingerol standard in 55:45(v/v) acetonitrile-water mobile phase.**

### 4.3.2 HPLC for determination of [6]-gingerol contents in *Z. officinale* rhizome extracts

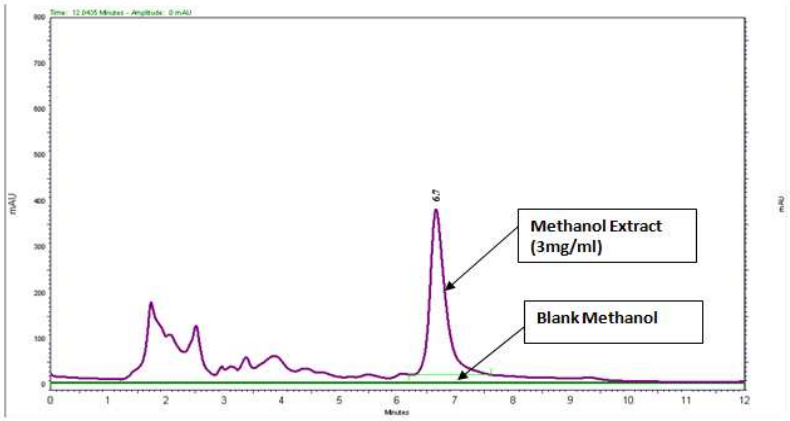
[6]-gingerol is the most abundant (80-90%) and important pungent compound among the other gingerols present in fresh and dry ginger products (Govindarajan, 1982). In this study, [6]-gingerol (Sigma Aldrich) at six different concentrations (25, 50, 100, 200, 400 and 800 µg/ml) was analyzed and peak area determined (average of triplicate injections). A calibration curve was plotted for concentration versus peak area. The result, by linear regression analysis, showed a very good linear relationship between peak area and concentration (figure 4.3). The correlation coefficient was $R^2$= 0.9975. [6]-gingerol content in ZOA, ZOM and ZOH was calculated using the standard curve (figure 4.3). Among four different extracts, ZOM showed high [6]-gingerol

content followed by ZOA and ZOH. [6]-gingerol was not detected in ZOC. A representative HPLC chromatogram of ZOA, ZOM and ZOH from three independent and triplicate quantifications is shown in figure 3. A, B, C and ZOA, ZOM and ZOH possessed 29.32±1.97, 137.32±1.97and 15.96±1.39mg [6]-gingerol content per gram of extract respectively (Table 4.5). Since ZOM and ZOA contained significant higher amount of total phenolic content (TPC) and [6]-gingerol contents, they were chosen for subsequent bioassays.

These data support earlier studies that identified [6]-gingerol as a component of *Z. officinale* rhizomes. [6]-gingerol was found to be one of the major constituents of *Z. officinale* rhizome extracts (Bao *et al*., 2010, Young *et al*., 2002). Similarly, supercritical $CO_2$ extraction of ginger was undertaken with two conditions of 200 bar at 35°C and 230 bar at 40° C and showed that the extracts from both conditions had [6]-gingerol contents of 238.94 ± 0.79 and 170.50 ± 0.45 mg/g extract and total phenolic contents of 183.96 ± 1.25 and 126.04 ± 0.72 mg gallic acid/g extract, respectively (Puengphian and Sirichote, 2008). In the study of Kubra *et al*., 2011, total polyphenols and [6]-gingerol contents were higher in methanol fraction compared to water fraction. The obtained results can be explained by the fact that phenolic compounds are distributed in investigated extracts as a function of polarity of applied solvents (Callste *et al*., 2001). In the investigation, methanol appeared the best solvent for extraction of phenolic acids and [6]-gingerol.




A.

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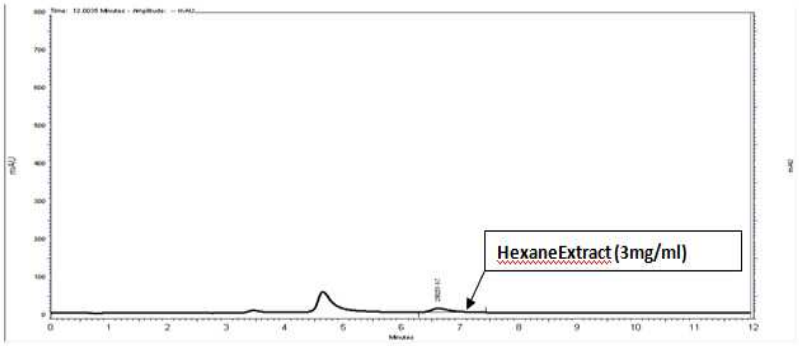



C.

**Fig 4.5 The typical HPLC chromatogram of *Z. officinale rhizome* extracts.** A) ZOA (3mg/mL). B) ZOM (3mg/mL). C) ZOH (3mg/ml) in 55:45(v/v) acetonitrile-water mobile phase.

**TABLE 4.5 Summary of retrieved dry weights of *Zingiber officinale* rhizome extracts using water, methanol, chloroform, and hexane as extraction solvents.**

| Extraction Solvent | Obtained Dry weight (g) | % Yield | Total Phenolic Contents mg GAE/g | [6]-gingerol Contents mg [6]-gingerol/g |
|---|---|---|---|---|
| Water (ZOA) | 4.790 | 7.98 | 68.17±0.28 | 29.32±1.97 |
| Methanol (ZOM) | 1.706 | 2.84 | 252.89±0.55 | 137.32±1.97 |
| Chloroform (ZOC) | 2.038 | 3.39 | 56.69±0.57 | - |
| Hexane (ZOH) | 0.410 | 0.68 | 47.15±0.42 | 15.96±1.39 |

**4.4 Cytotoxic potential of *Z. officinale* rhizome extracts (ZOA, ZOM) and pure [6]-gingerol**

The cytotoxic effects of ZOA, ZOM and pure [6]-gingerol on Vero and MDCK cells were determined using an MTT assay. All extracts were filter-sterilized with 0.22 µm membrane filter prior to use for determining median Inhibitory Concentration ($IC_{50}$) of extracts.The MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product (Appendix F.1). The cells are solubilized with an organic solvent (eg. DMSO) and the released, solubilized formazan reagent are measured spectrophotometrically. Since reduction of MTT

can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

The $IC_{50}$ value of ZOA, ZOM and pure [6]-gingerol for Vero cells was 348.8 µg/mL, 221.5µg/mL and 199.9µg/mL respectively (Table 4.6; figure 4.6 A, B and C). Similarly, the IC50 value of ZOA, ZOM and pure [6]-gingerol for MDCK cells was 368.1µg/mL, 186.4µg/mL and 150.2µg/mL respectively (Table 4.6; figure 4.7 A, B and C). The assay demonstrated that 50µg/mL ZOM 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. As a postitive control for cytotoxicity, bleomycin sulfate, on the other hand, displayed potent activity against Vero and MDCK cells as no sign of viability was observed after 72 hours.

**Table 4.6 In vitro cytotoxicity effects of *Z. officinale* rhizome extracts and [6]-gingerol against Vero and MDCK cells.**

| **Test Extracts** | **ZOA** | | **ZOM** | | **[6]-gingerol** | |
|---|---|---|---|---|---|---|
| Cells | Vero | MDCK | Vero | MDCK | Vero | MDCK |
| $IC_{50}$ (µg/mL) | 348.8 | 368.1 | 221.5 | 186.4 | 199.9 | 150.2 |
| $R^2$ | 0.9910 | 0.9878 | 0.9879 | 0.9858 | 0.9861 | 0.9925 |

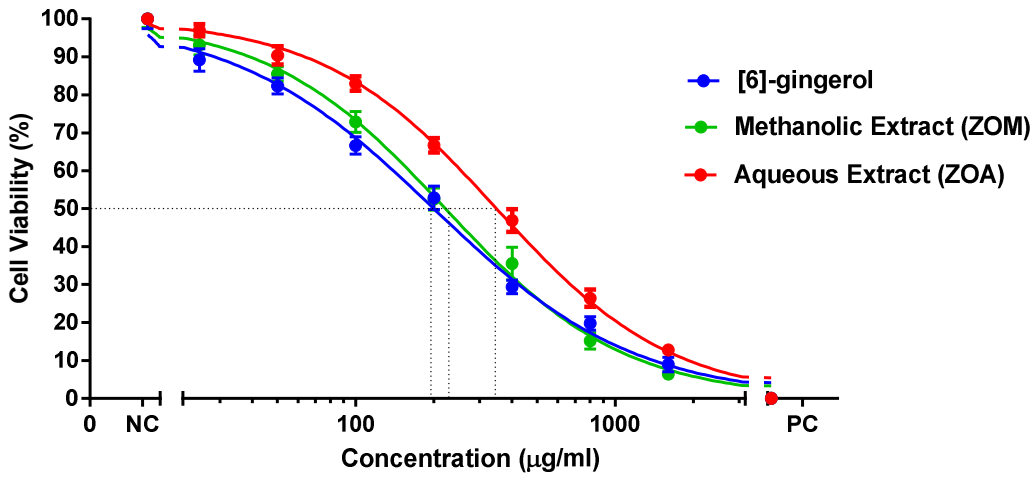



**Fig 4.6 Cytotoxicity of *Z. officinale* rhizome extracts and [6]-gingerol on Vero cells.** MTT assay was used to evaluate the cytotoxicity ($IC_{50}$) of the extracts and pure [6]-gingerol. All experiments were conducted in triplicates.

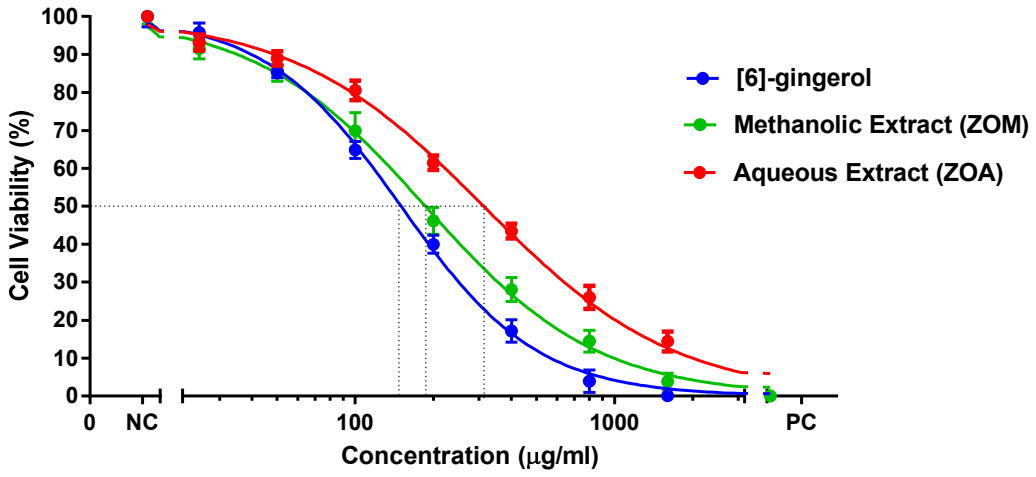



**Fig 4.7 Cytotoxicity of *Z. officinale* rhizome extracts and [6]-gingerol on MDCK cells.** MTT assay was used to evaluate the cytotoxicity ($IC_{50}$) of the extracts and pure [6]-gingerol. All experiments were conducted in triplicates.

**4.5 Propagation of Dengue Virus and Viral Infectivity Titration by Foci Formation Assay**

Clinical isolates of dengue virus serotype -3 (DV3-strain SLMC-50) were propagated in monolayers of C6/36 cells cultured in 25cm$^2$ tissue culture for 14 days at 28$^0$C in the absence of $CO_2$. Infected culture fluid (ICF) was collected on days 7 and 14. Strain SLMC-50 was chosen as it was derived from a clinical case of severe dengue and strains of serotype DV3 have been found to cause more severe illness and more predominant in past infections in the Philippines ( Ypil-Butac*et al*., 2006; Capeding*et al*., 2010; Bomasang and Suzara-Masaga, 2008; Libraty *et al*., 2009, Alera, 2009).

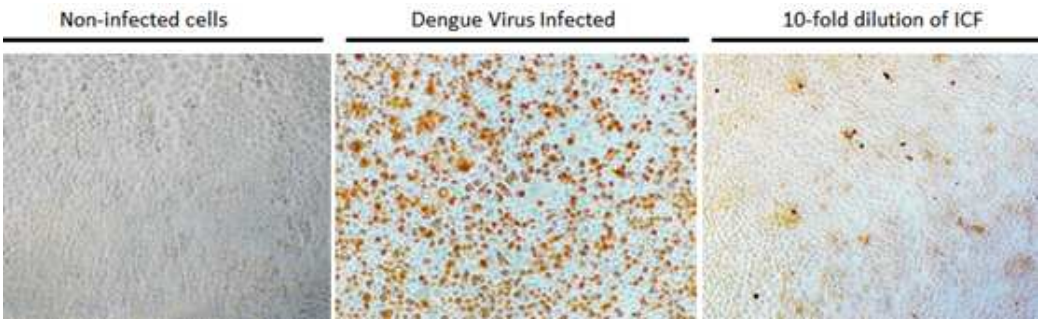



**Fig 4.8 Determination of infectivity titers of propagated dengue virus (DV3 SLMC-50 isolate).** Infection foci of DV in a monolayer of BHK-21 cells were detected through immunostaining using monoclonal antibodies against the virus (clone 6B9). Infectivity titers were expressed in focus forming units per mL (FFU mL$^{-1}$).

The titer of infectious particles was quantified and reported as foci-forming units per mL (ffu/mL). Immunoperoxidase staining was performed to

detect and quantify dengue virus infectivity titer in BHK-21 [C-13] cells. For the propagated DV3 (SLMC-50) isolate, infectivity titer was found to be 6.9 x $10^7$±0.07 ffu/mL.

**4.6 Induction of MMPs on Vero cells by DV3 infection and determination of** MMPs **activity by Gelatin Zymography Assay**

**4.6.1. Infection of Vero cells with DV3 and collection of the conditioned media**

The conditioned cell culture media from dengue-infected Vero cells (which were adapted in serum free MEM) was collected in a sterile 15mL conical tube. The removal of virus particles was done by using size exclusion ultrafiltration using Amicon$^{TM}$-4 Centrifugal Filter Units (Millipore) with molecular cut off 100kDa and MMPs were concentrated by centrifugation using Amicon$^{TM}$-4 Centrifugal Filter Units (Millipore) with molecular cut off 30kDa.

Reverse transcription–PCR was performed to ensure the absence of dengue viral nucleic acids from the collected conditioned media from DV3 infected Vero cells after Amicon filtration (figure 4.9). Virus-free cell conditioned media containing the soluble factors like MMPs produced 24h after viral washing out, that is, 4h after DV3 infection of Vero cells were used for all experiments.

**4.6.2 PCR detection for absence of viral genome from collected culture supernatant**

Thus, collected concentrated conditioned media were subjected to PCR detection to confirm the absence of viral genome after ultrafiltration and all were found to be free of viral genome as no band was seen in the agarose gel at the expected size of 511bp (figure 4.9). We observed a band at 511bp for the positive control while no band seen for negative control. After confirmation of the absence of viral genome in the collected concentrated conditioned media through PCR detection, conditioned media were subjected to further bioassays.

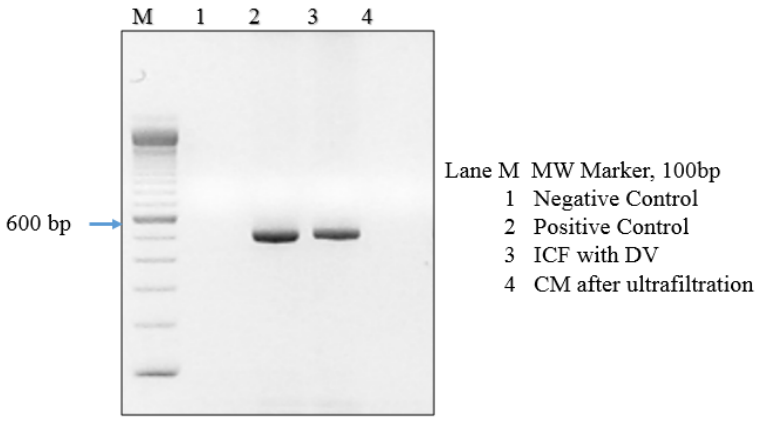
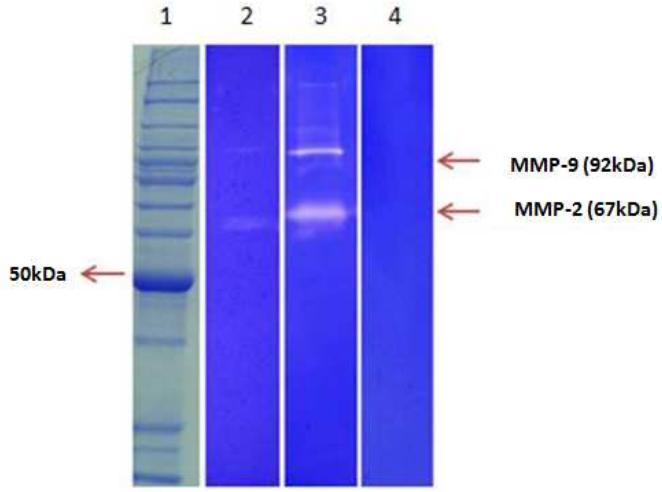



**Fig 4.9 Absence of viral genome in collected concentrated CM.** Total RNA from CM was purified and reverse transcribed using random hexamer primers. Dengue consensus primers DC-1: 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' and DC-2: 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3' spanning the c-prM region of the dengue virus genome was used to detect and amplify viral RNA by reverse transcription-PCR. The absence of the 511bp band indicates absence of DV.

**4.6.3 DV infection of Vero cells triggers the 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, to a lesser extent, MMP-9 at 92 kDa (band corresponding to the molecular weight of pro-MMP-9) ($p<0.0001$ compared to non-infected cells). This enhancement of proteolytic activity was reduced after treatment with the Ethylenediaminetetraacetic Acid (EDTA, a known metalloproteinase inhibitor) (figure 4.10). These data verified that these enzymes are metalloproteinases as these proteinases are dependent upon divalent cations for their optimal activity, and hydrolyse gelatin. The relative molecular mass of gelatinolytic MMPs was determined by comparison with a molecular weight protein marker in the adjacent lane. Gelatinase proteins that migrate through 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 (Troeberg and Nagase, 2004; Shimokawa*et al*., 2002).

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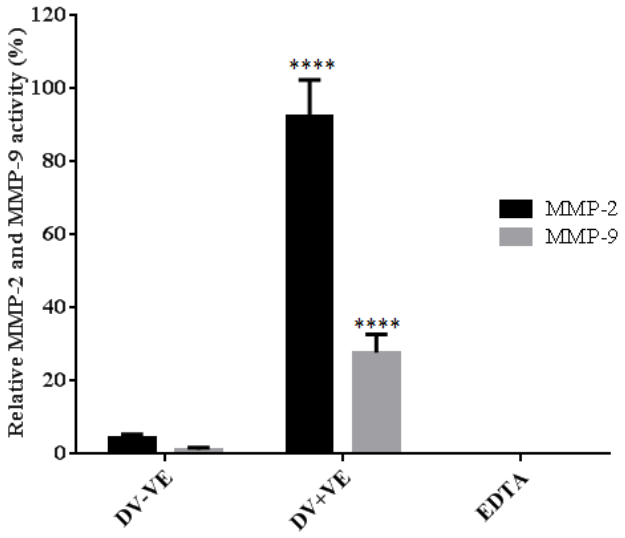



B.

**Fig 4.10 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 (4). Concentrated CM from DV infected cells treated with 10mmol/l EDTA. (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.

This study had shown increased activities of MMP-2 and to a lesser extent MMP-9 in conditioned media collected from DV infected Vero cells compared to non-infected cells which is in agreement with the observations of Luplerdlop *et al*., (2006), who demonstrated that DV-infected cell supernatants had significantly increased level of MMP-2 and MMP-9 as compared to non-infected cell supernatants. Another study conducted by Luplerdlop and Misse, (2008), had demonstrated that DV-infected human macrovascular endothelial cells stimulated the overproduction of MMP-2 and MMP-9 causing the disruption of vascular endothelium-cadherin cell-cell adhesion and further enhanced endothelial permeability. Similarly, Seanpomng *et al*., (2015) also found that DV-infected hepatocytes (HepG2 cells) increased the levels of MMP-2 while infected monocytes (U937 cells) promoted MMP-9 production. Significant elevation of circulating MMP-9 and MMP-2 in dengue patients and association with the disease severity and plasma leakage compared to healthy controls is well established in clinical investigation of DV infection (Kubelka *et al*., 2010; Weg *et al*., 2014).

Remodelling of the extracellular matrix (ECM) occurs in various physiological processes (post-partum uterine involution, ovulation, embryogenesis, odontogenesis, bone remodelling, cutaneous healing etc.); consequently, synthesis and destruction of ECM is strictly regulated (Bourd-Boittin *et al*., 2004). MMPs are the main protease family involved in this

regulation. MMP-2 and MMP-9 (gelatinases A and B, respectively) are known to play key roles in tissue remodeling and repair through the degradation of many matrix proteins. The activity of MMP-2 and MMP-9 is regulated, respectively, by tissue inhibitors of metalloproteinases termed TIMP-2 and TIMP-1 (Gomez *et al.,* 1997). Control over MMP and/or TIMP activity *in vivo* occurs at different levels and involves factors such as regulation of gene expression, activation of zymogens and inhibition of active enzymes by specific inhibitors. Many MMPs and TIMPs are regulated at the level of transcription by a variety of growth factors, cytokines and chemokines e.g. (Yan & Boyd, 2007). The imbalance in MMPs/TIMPs ratio is involved in the development of diseases such as arthritis, cancer cell invasion, metastasis, and fibrosis (Nagase, 1996; Woessner, 1994). Matrix metalloproteinases and their inhibitors, tissue inhibitors of metalloproteinases are thought to contribute to the pathogenesis of severe dengue via their influence on the integrity of the vascular endothelium leading to the vascular leakage (Luplerdlop*et al*., 2006; Luplerdlop and Misse, 2008).

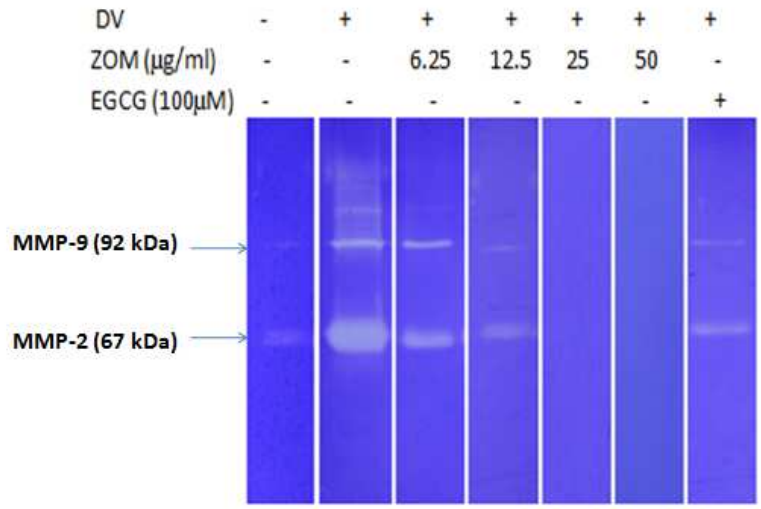
**4.7 Inhibitory effects of *Z. officinale* rhizome extracts (ZOA, ZOM) and [6]-gingerol on DV induced MMP-2 and MMP-9 activities**

Balance between the synthesis of the active forms of MMPs and their inhibition by TIMPs control the proteolytic actions of MMPs in normal physiological conditions whereas in pathological process proteolytic activity of

MMP is increased that degrade proteins and proteoglycans leading to a loss of tissue integrity which ameliorate the various disease conditions like osteoarthritis and tumor metastasis (De *et al*., 1999). Therefore, it is of great significance to develop MMP inhibitors which might control the abnormal regulation of MMP expression, secretion and their activities.

In this study, the proteolytic activities of MMP-2 and MMP-9 in CM collected from DV-infected Vero cells were decreased by ZOA, ZOM and pure [6]-gingerol treatment in a dose-dependent manner (Figure 4.14). There is also a significant difference in the mean percentage inhibition over of four concentrations ($p<0.001$) in which multiple comparison analysis indicated that the MMP-2 and MMP-9 inhibitory activities was greater when treated with the concentrations of 25µg/mL and 50µg/mL compared to groups treated with 6.25µg/mL and 12.5µg/mL. There was complete inhibition of MMP-2 and MMP-9 activities at higher concentrations of ZOM (25 and 50µg/mL). MMP-2 activity was reduced significantly to 80.66% ($p=0.0004$) and 59.33% ($p<0.0001$), in response to ZOM-treatment with concentrations of 6.25µg/ml and 12.5 µg/ml respectively. Similarly, the activity of MMP-9 was significantly reduced to 84.67% ($p=0.0033$) and 65.0% ($p<0.0001$) in response to ZOM-treatment with 6.25 and 12.5 µg/mL respectively (figure 4.11). MMP-2 and MMP-9 activities were reduced to 34.3% and 28.5% ($p<0.0001$) respectively with 100 µM epigallocatechin gallate (EGCG) treatment. EGCG is a main constitute of green

tea polyphenols and was used as a control inhibitor of MMP activity as it as been shown to cause strong inhibition of gelatinolytic activities of MMP-2 and MMP-9 and of elastolytic activity of MMP-12 (Demeule *et al.*, 2000).

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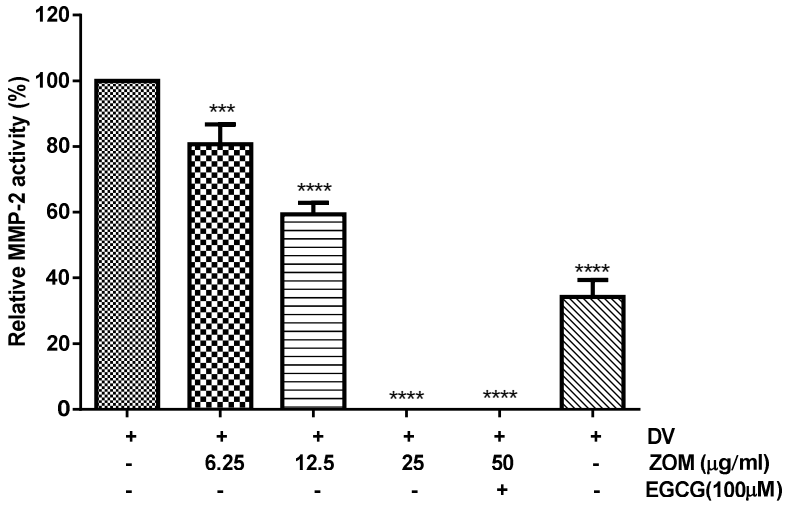


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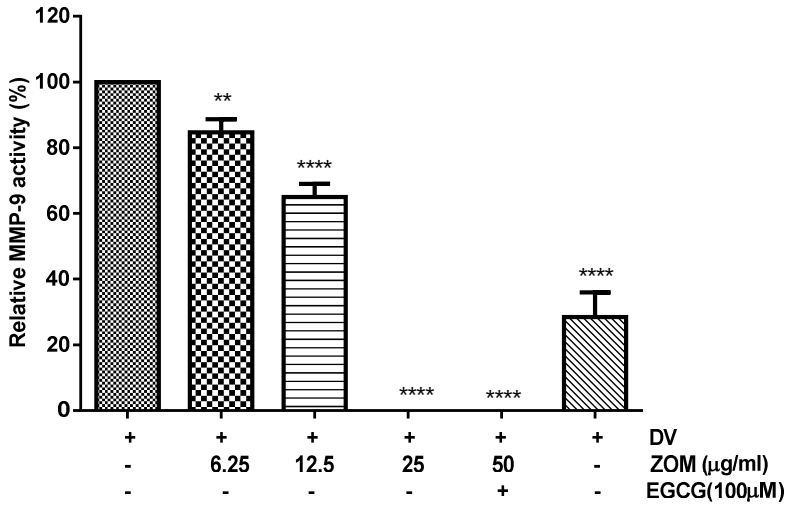
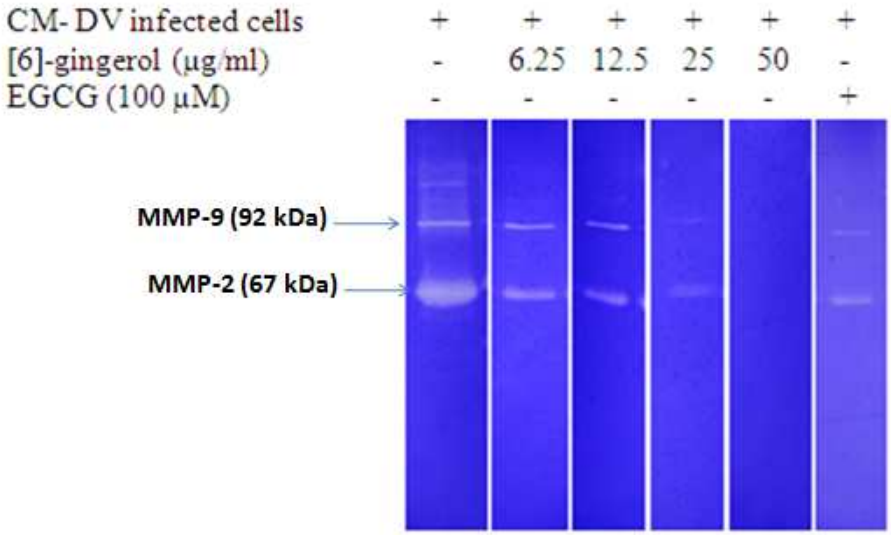



C.

**Fig 4.11 Effect of ZOM on the activity of MMP-2 and MMP-9 in CM collected from DV infected Vero cells (m.o.i=1ffu/cell).** Dose dependent inhibition of MMP-2 and MMP-9 activity after treatment with ZOM. EGCG was used as control MMP inhibitor. 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), $p<0.05$ (*), $p<0.01$(**), $p<0.001$(***) and $p<0.0001$ (****).

The results revealed that the activities of MMP-2 and MMP-9 in CM collected from DV infected Vero cells were decreased after treatment with [6]-gingerol in dependent of concentration, with a complete inhibition achieved at 50μg/ml (figure 4.12). MMP-2 activity was reduced significantly to 90.53% ($p=0.0157$), 87.06% ($p=0.0016$), 21% ($p<0.0001$) in response to [6]-gingerol treatment with concentrations of 6.25, 12.5 and 25μg/mL respectively. Similarly MMP-9 activity was reduced to 95.7%, 89%, 42.03% with concentrations of 6.25, 12.5 and 25μg/mL respectively. Here, 50μg/mL has the highest mean percentage

inhibition ($p<0.0001$) as compared to the other concentrations. MMP-2 and MMP-9 activities were reduced to 34.3and 28.5% ($p<0.0001$) respectively with 100µM EGCG treatment.

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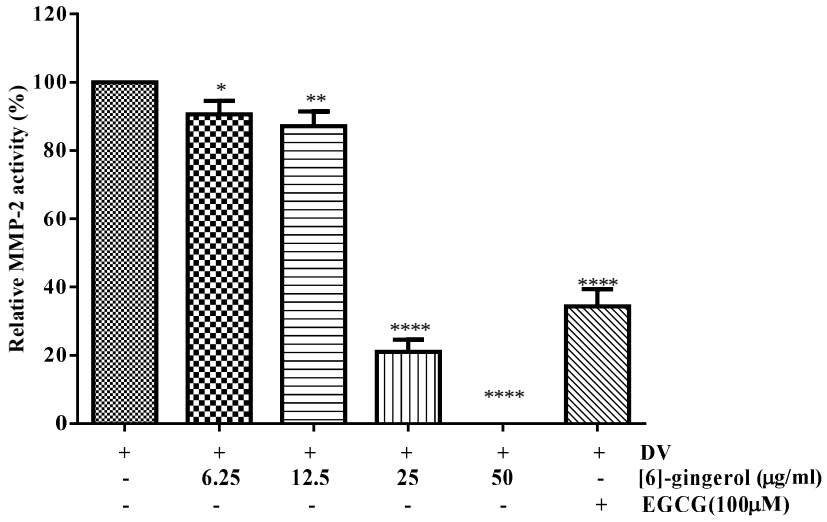


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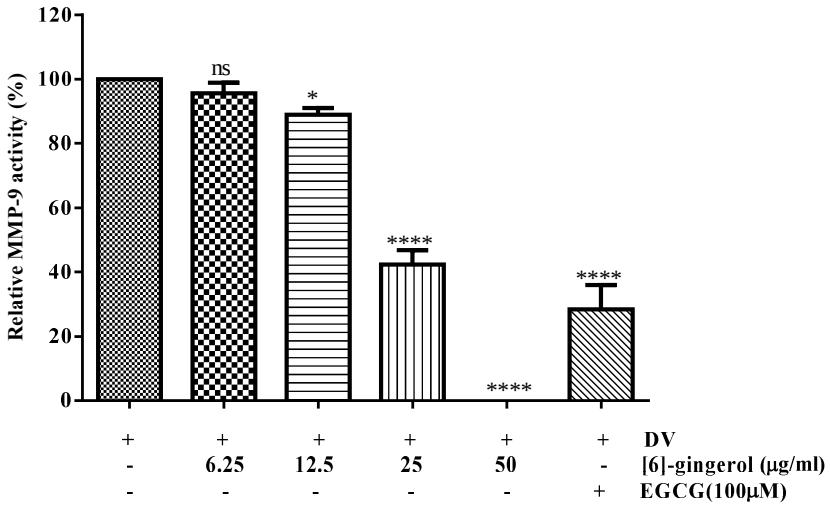
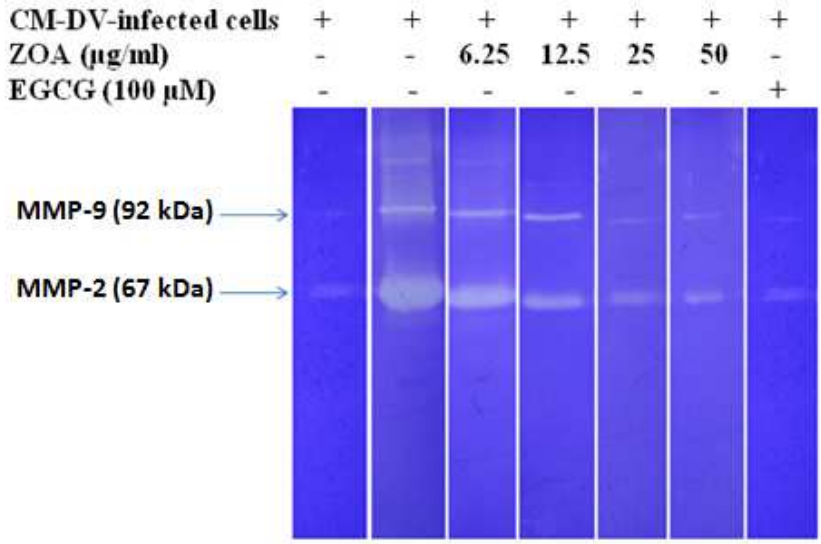



C.

**Fig 4.12 Effect of [6]-gingerol on the activity of MMP-2 and MMP-9 in CM collected from DV infected Vero cells (m.o.i=1ffu/cell).** Dose dependent inhibition of MMP-2 and MMP-9 activity after treatment with ZOM. EGCG was used as control MMP inhibitor. 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), $p<0.05$ (*), $p<0.01$(**) and $p<0.0001$ (****).

At the ZOA concentration of 6.25µg/mL treatment, no difference was observed in the MMP-2 activity compared to the untreated CM. MMP-2 activity was reduced significantly to 88.67% ($p=0.0243$), 38.33 and 16.33% ($p<0.0001$) in response to ZOA-treatment with concentrations of 12.5, 25 and 50µg/mL respectively. Similarly, there was no difference observed in MMP-9 activity at CM treated with 6.25 and 12.5µg/mL of ZOA while the activity of MMP-9 was significantly reduced to 58.33 % and 34.00% ($p<0.0001$) in response to ZOA-treatment with 25 and 50µg/mL respectively (figure 4.13). Unlike in ZOM and

pure [6]-gingerol treatment with their higher concentrations, there were never complete inhibition of the activities of MMP-2 and MMP-9 after treatment with the ZOA. MMP-2 and MMP-9 activities were reduced to 34.3% and 28.5% ($p<0.0001$) respectively with 100µM EGCG treatment.

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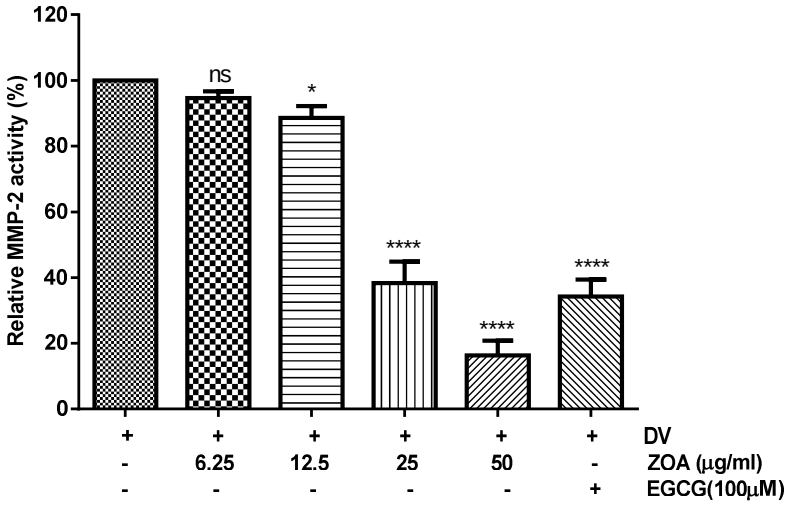


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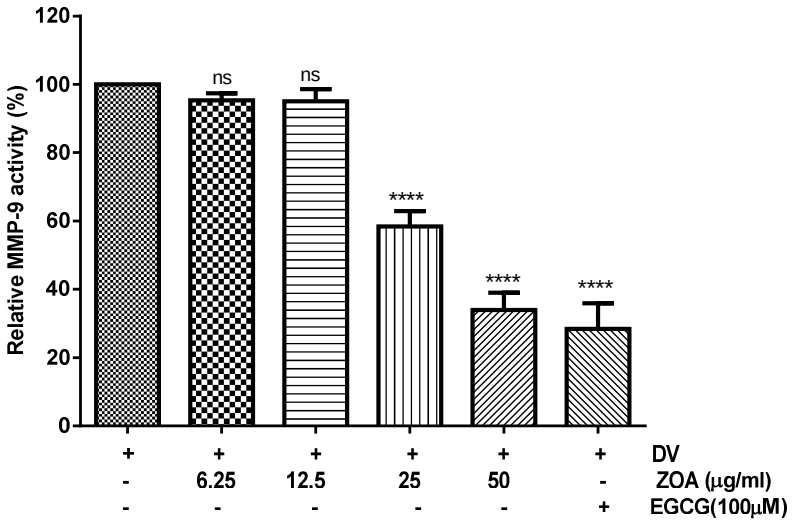



**Fig 4.13 Effect of ZOA on the activity of MMP-2 and MMP-9 in CM collected from DV infected Vero cells (m.o.i=1ffu/cell).** Dose dependent inhibition of MMP-2 and MMP-9 activity after treatment with ZOM. EGCG was used as control MMP inhibitor. Photograph of the MMP bands, which are representative of 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), $p<0.05$ (*), $p<0.01$(**) and $p<0.0001$ (****).

The results from this study show that there is a significant difference in the mean percentage inhibition of MMP-2 and MMP-9 activities of ZOA, ZOM and pure [6]-gingerol ($p<0.0001$) at their higher concentrations, in which Tukey's HSD post hoc analysis indicated that ZOM significantly exerts the highest mean percentage inhibition of MMP-2 and MMP-9 activities, followed by [6]-gingerol, while the ZOA do not differ significantly at its lower concentration. The effect of ZOM, pure [6]-gingerol and ZOA exert a concentration dependent decrease in the activities of MMP-2 and MMP-9 (figure 4.14). Comparing the values between

ZOA, ZOM and pure [6]-gingerol (Sigma), it was found that the activities of MMP-2 and MMP-9 in ZOM treatment were significantly lower than in pure [6]-gingerol (sigma) and ZOA treatment. The anti-MMPs activities of ZOM and ZOA have been attributed to their naturally occurring compounds such as gingerols, shogaols etc. that may prevent vascular leakage caused by overproduced MMPs induced by DV-infection. One possible mechanism by which ZOA, ZOM and pure [6]-gingerol inhibits MMPs may be due to their ability to chelate minerals such as iron and zinc. All native MMPs contain a catalytic domain with highly conserved zinc and calcium binding sites. Zinc ion is necessary for enzyme activation, whereas calcium stabilizes enzyme structure (Kahari and Saarialho-Kere, 1999).

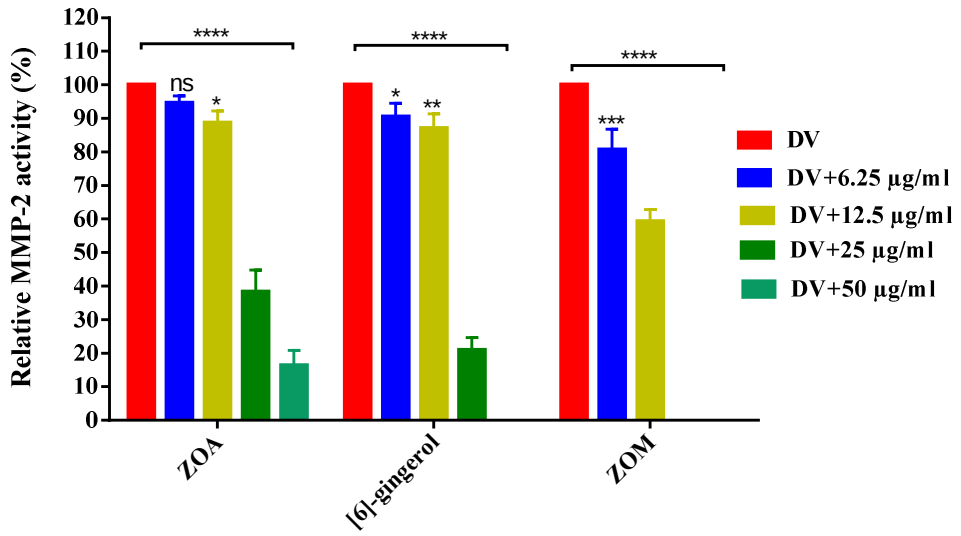
**Table 4.7 Relative MMP-2 and MMP-9 activities in CM collected from DV-infected Vero Cells after 24 hours post-treatment with EGCG (100 µM), ZOM, ZOA and [6]-gingerol at different Concentrations.**

| MMPs | Treatment | Mean MMPs Activity (%) | SD | SEM |
|---|---|---|---|---|
| MMP-2 | CM-DV (Untreated) | 100.00 | 0.00 | 0.00 |
| | CM-DV+ZOM 6.25 µg/mL | 80.67 | 6.11 | 3.53 |
| | CM-DV+ZOM 12.5 µg/mL | 59.33 | 3.51 | 2.03 |
| | CM-DV+ZOM 25 µg/mL | 0.00 | 0.00 | 0.00 |
| | CM-DV+ZOM 50 µg/mL | 0 .00 | 0.00 | 0.00 |
| | CM-DV+[6]-gingerol 6.25 µg/mL | 90.53 | 4.00 | 2.30 |
| | CM-DV+[6]-gingerol 12.5 µg/mL | 87.06 | 4.40 | 2.50 |
| | CM-DV+[6]-gingerol 25 µg/mL | 21.00 | 3.60 | 2.10 |
| | CM-DV+[6]-gingerol 50 µg/mL | 0.00 | 0.00 | 0.00 |
| | CM-DV+ZOA 6.25 µg/mL | 94.67 | 2.10 | 1.20 |
| | CM-DV+ZOA 12.5 µg/mL | 88.67 | 3.5 | 2.0 |
| | CM-DV+ZOA 25 µg/mL | 38.33 | 6.5 | 3.8 |

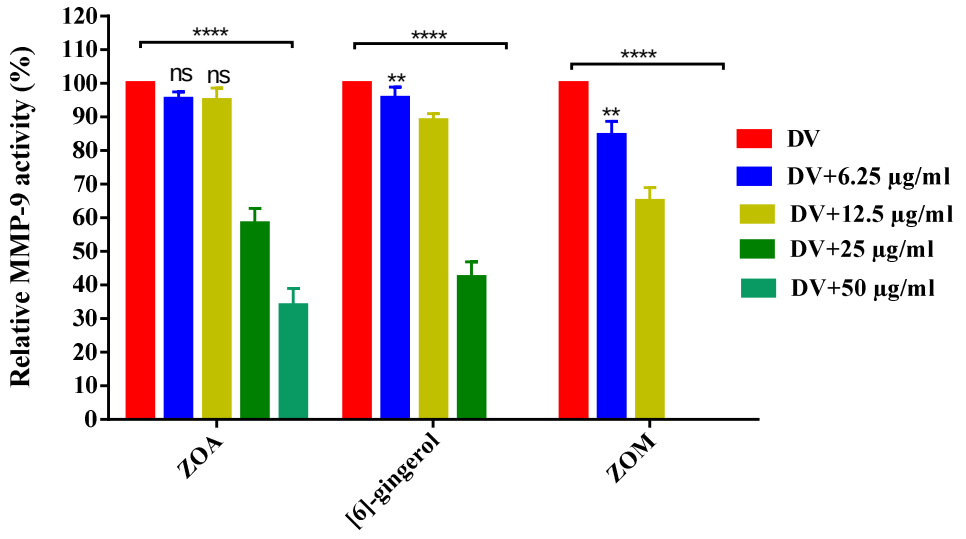
| | | | | |
|---|---|---|---|---|
| | CM-DV+ZOA 50 µg/mL | 16.33 | 4.5 | 2.6 |
| | CM-DV+EGCG 100 µM | 34.30 | 5.12 | 2.96 |
| MMP-9 | CM-DV (Untreated) | 100.00 | 0.00 | 0.00 |
| | CM-DV+ZOM 6.25 µg/mL | 84.67 | 4.04 | 2.33 |
| | CM-DV+ZOM 12.5 µg/mL | 65.00 | 4.00 | 2.31 |
| | CM-DV+ZOM 25 µg/mL | 0.00 | 0.00 | 0.00 |
| | CM-DV+ZOM 50 µg/mL | 0.00 | 0.00 | 0.00 |
| | CM-DV+[6]-gingerol 6.25 µg/mL | 95.67 | 3.21 | 1.86 |
| | CM-DV+[6]-gingerol 12.5 µg/mL | 89.00 | 2.00 | 1.12 |
| | CM-DV+[6]-gingerol 25 µg/mL | 42.03 | 4.51 | 2.60 |
| | CM-DV+[6]-gingerol 50 µg/mL | 0.00 | 0.00 | 0.00 |
| | CM-DV+ZOA 6.25 µg/mL | 95.33 | 2.08 | 1.20 |
| | CM-DV+ZOA 12.5 µg/mL | 95.00 | 3.61 | 2.08 |
| | CM-DV+ZOA 25 µg/mL | 58.33 | 4.51 | 2.60 |
| | CM-DV+ZOA 50 µg/mL | 34.00 | 5.00 | 2.89 |
| | CM-DV+EGCG 100 µM | 28.45 | 7.50 | 4.33 |

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 (Weng *et al*., 2010; Solanki *et al*., 2011).The data of this study suggest that ginger extracts and [6]-gingerol have anti-MMPs activities and are supported by the findings of previous studies. 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. Weng *et al*., (2010) showed that [6]-gingerol and [6]-shogaol exerted anti-invasive activity against hepatoma cells through regulation of MMP-9 and TIMP-1 in a dose dependent manner. Another study by Lee *et al*., (2008) demonstrated that [6]-gingerol inhibited metastasis accompanying with dose dependent inhibition of cell adhesion, invasion, motility

and activity of MMP-2 and MMP-9 in MDA-MB-231 human breast cancer cells. These reports supported the findings of this study that the natural compound [6]-gingerol plays an important anti-MMP role. This study is the first of its kind that has proven that that ginger extracts and [6]-gingerol treatment could modulate the activities of gelatinolytic MMP-2 and MMP-9 in CM collected from DV-infected Vero cells.




A.




B.

**Fig 4.14 Effects of ZOA, [6]-gingerol and ZOM on the activity of MMP-2 and MMP-9 in CM collected from DV infected (m.o.i=1ffu/cell) Vero cells.** Dose dependent inhibition of MMP-2 and MMP-9 activity after treatment with ZOA, [6]-gingerol and ZOM as demonstrated by gelatin zymography.Quantitative analysis of the bands for MMP-2 (A) and MMP-9 (B). Each bar represents the mean ± S.D. calculated from three independent experiments are indicated by not significant (ns), $p<0.05$(*), $p<0.01$(**), $p<0.001$ (***) and $p<0.0001$ (****).

### 4.8. Gene Expression Assays

This study has demonstrated a decrease in the protease activity of MMP-2 and MMP-9 after treatment with ZOM, ZOA and pure [6]-gingerol. Thus, to further evaluate the effect of extracts and pure [6]-gingerol (6.25 µg/mL, 12.5 µg/mL, 25 µg/mL and 50 µg/mL concentrations) on the mRNA expression of MMP-2, MMP-9 and their natural tissue inhibitors TIMP-1, TIMP-2 following DV3-infection, real-time quantitative RT-PCR was performed using relative quantitation by the comparative CT method using the gene for Glyceraldehyde-

3-Phosphate Dehydrogenase (GAPDH) as an internal control. Cells receiving no extract were mock-inoculated with media alone and served as a negative control. The experimental control group was treated with EGCG. At 48 hours post-treatment, changes in the mRNA levels were detected as compared to the negative control (non-infected cells) and positive control (DV-infected cells).

*Z. officinale* rhizome extracts and pure [6]-gingerol were added to DV-infected Vero cells to test whether they can modulate, inhibit or block overexpression of MMPs and enhance the expressions of TIMPs as induced by DV3 infection. Differences were observed in the ability of ZOA, ZOM and pure [6]-gingerol to reduce the mRNA expression levels of DV-stimulated overexpression of MMP-2 and MMP-9 while enhancing the mRNA expression of their natural inhibitors, TIMP-1 and TIMP-2. As expected, DV-infection of cells alone, without inhibitors, resulted in increased expression of gelatinolytic matrixmetalloproteinases and their natural tissue inhibitors when compared to non-infected cells.

**4.8.1. DV infection of Vero cells triggers the overexpression of MMP-2, MMP-9, TIMP-1 and TIMP-2**

Using real time RT-PCR, this study found that MMP-2 and MMP-9 gene expression were upregulated in Vero cells upon DV infection ($p<0.0001$) (Figure 4.15 A, B). DV infection of Vero cells also upregulated the expression of TIMP-1

(p<0.0001) and TIMP-2 (p<0.0001) after 4 h post-infection following 48 h of incubation (Figure 4.15 C, D). Here DV infection induced increased expression of MMP-2 by 9.43±0.91 folds times higher than the expressed level of MMP-2 in uninfected Vero cells (Figure 4.15, A). Similarly MMP-9 was also increased but to a lesser extent, 5.85±0.40 folds higher, than MMP-2 in DV-infected Vero cells compared to uninfected cells (Figure 4.15, B). Likewise, TIMP-2 was more expressed than TIMP-1, though both TIMP-2 and TIMP-1 were significantly more expressed in DV-infected Vero cells compared to un-infected cells. For TIMP-2 mRNA was, 3.33± 0.21 folds higher and TIMP-1 mRNA was 1.24±0.031 folds higher in DV-infected cells compIUODared to uninfected cells (figure 4.16, C and D).

The findings of this study were in agreement with the observations of Luplerdlop *et al*., (2006) and Luplerdlop and Misse, (2008), who demonstrated that DV-infected cells had significantly overexpressed MMP-2, MMP-9 and their natural tissue inhibitors TIMP-1 and TIMP-2 as compared to non-infected cells. 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. Interestingly, this 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 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. Overall MMP activity is based on balance between levels of activated MMPs and free TIMPs. Although each TIMP appears capable of inhibiting several MMPs, these proteins exhibit preferential inhibitory capacity; *e.g.,* TIMP-1 and -2 selectively inhibit MMP-9 and -2, respectively (Baker *et al*., 2000). Most of the MMPs are inhibited by prototypic inhibitor, TIMP-1; and is present in various cell types (Lacraz *et al*., 1995); however, TIMP-1 has inhibitory activity against MMP-9 (Figueira *et al*., 2009). TIMP-2 inhibits proMMP-2 10-fold more effectively than TIMP-1 (Stetler-stevenson *et al*., 1989; Howard *et al*., 1991). 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 (Kinoshita *et al*., 1998).

Since the MMPs possess extremely potent proteolytic activity, their activity and expression is regulated at many levels. A large portion of MMP regulation takes place at the transcriptional level. MMP transcription is under the control of a number of factors such as activating protein (AP)-1, E26 transformation-specific translocation variant (ETV)-4, nuclear factor (NF)-κB (Yan and Boyd, 2007) and hypoxia-inducible factor (HIF)-1α (Higashida *et al*., 2010). A feature common to most MMPs is that they are inducible. MMP gene expression can be enhanced or suppressed by many factors. Examples of inducers of MMP gene expression are cytokines, growth factors, phorbol esters, actin disrupters,

physical stress and oxidative stress (Nagase and Woessner, 1999; Takimoto*et al*., 2005). Factors such as interleukins, interferons, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) can all increase expression of MMPs through activation of the AP-1 or the ETV-4 elements (Yan and Boyd, 2007).

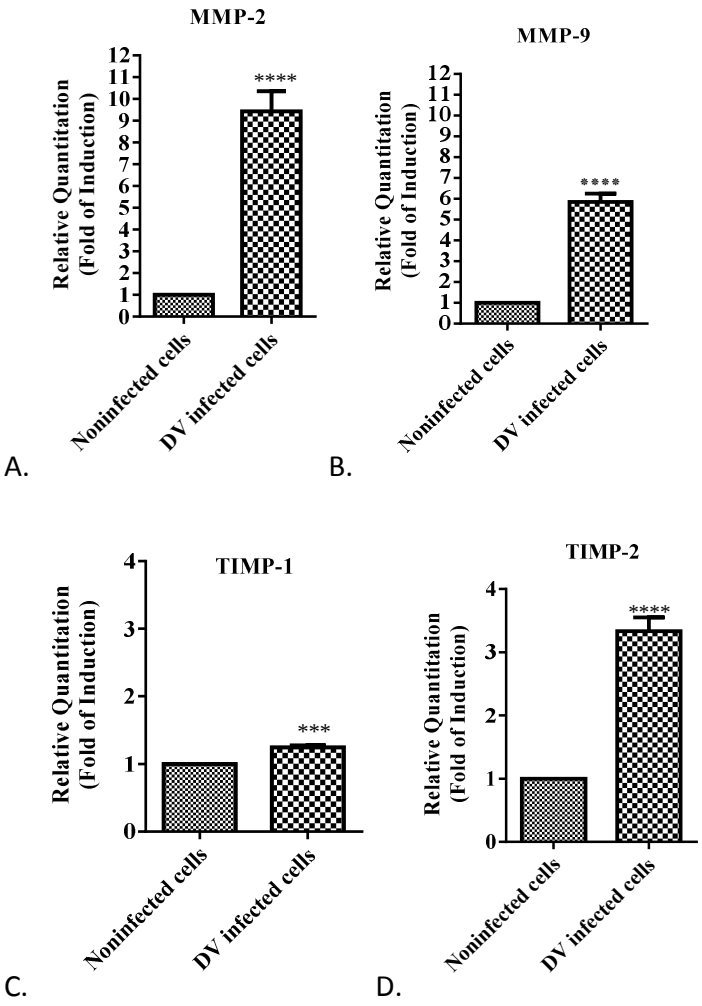



**Fig 4.15 Induction of gelatinolytic MMPs and their tissue inhibitors TIMPs gene expression on DV-infection of Vero cells**. Noninfected cells and DV-infected cells were collected after at 4 h post-infection following 48 h of incubation to assess the expression of MMP-2 and MMP-9 transcripts by Real time RT-PCR using specific primers. GAPDH was used as a positive control. Data are representative of three independent and duplicate experiments. Statistical

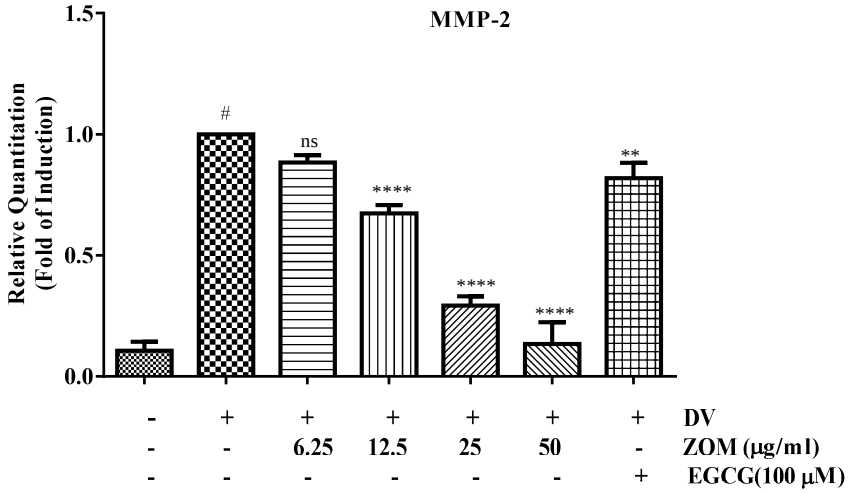
significance p<0.001 (***), P<0.0001 (****) was determined from three independent and duplicate experiments using Student's t-test.

**4.8.2. ZOA, ZOM and pure [6]-gingerol transcriptionally regulates MMP-2, MMP-9, TIMP-1 and TIMP-2**

With an increased concentration of ZOM the expressions of TIMP-1 and TIMP-2 were enhanced along with decreased expressions of MMP-9 and MMP-2 respectively, whereas that of the internal control (GAPDH) remained unchanged (figure 4.16). At the ZOM concentration of 6.25µg/mL no difference was observed in the MMP-2 gene expression level. 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 12.5µg/mL, 25µg/ml and 50µg/mL respectively (figure 4.16, A). The expression of MMP-9 was significantly down-regulated to 0.81 (P=0.0004), 0.71, 0.47 and 0.39 (p<0.0001) in response to ZOM treatment of 6.25, 12.5, 25 and 50 µg/ml respectively (figure 4.16, B).

There was a significant increase in the expression level of the TIMP-1 gene in the ZOM treated DV infected cells with 6.25, 12.5, 25 and 50µg/ml to 1.26 (P=0.0079), 2.92, 3.12 and 3.84 folds (P< 0.0001) respectively (figure 4.16, C). Similarly, expression of the 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 50µg/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 DV infected cells

(figure 4.16, D). This is the first study which demonstrated that ZOM significantly downregulated the expression of MMP-2 and MMP-9 and upregulated the expression of TIMP-1 and TIMP-2 in DV infected cells.

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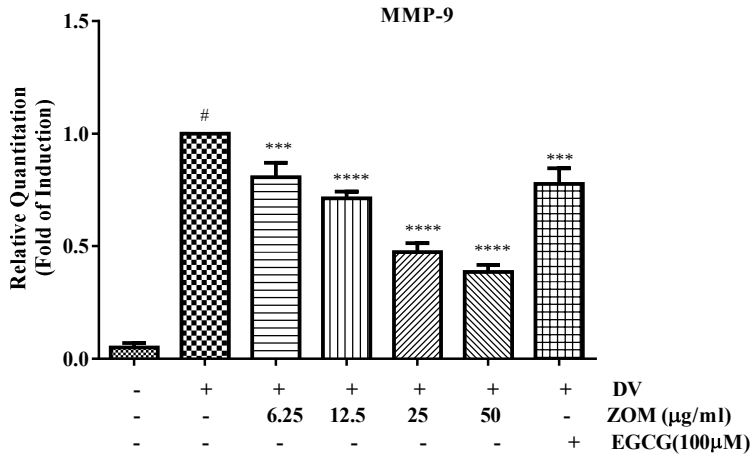


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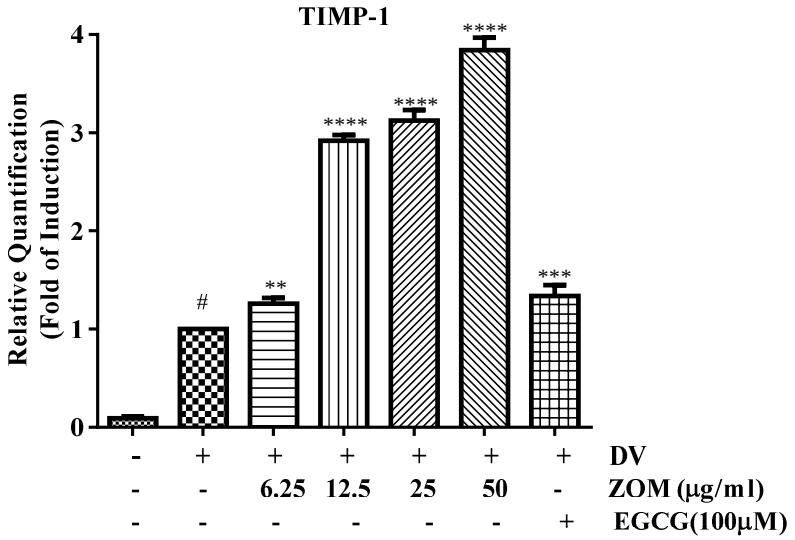
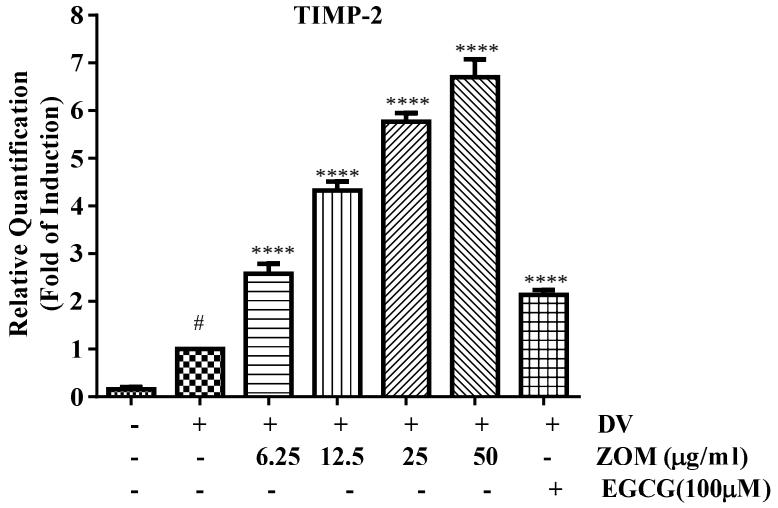



C.




D.

**Fig 4.16 Concentration-dependent inhibitory effects of ZOM 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 to form a monolayer following infection with DV and treatment with various concentrations of ZOM and 100µM EGCG for 48 hours at 37°C. For quantitative analysis, total RNA was isolated and RT followed by real time PCR was performed to investigate the gene expression level. Each bar

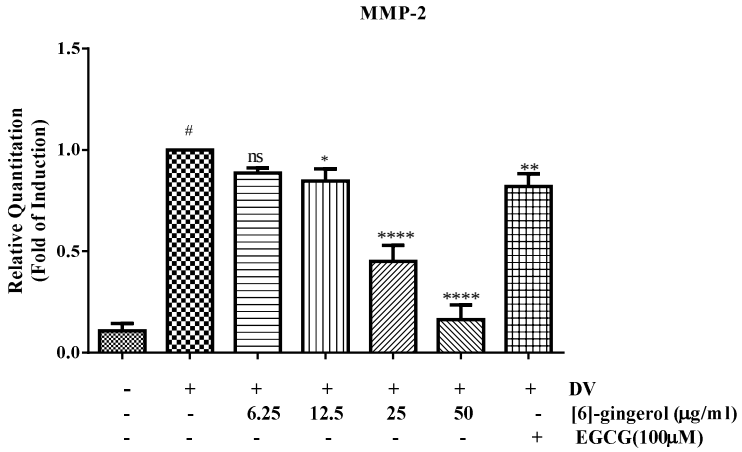
represents the mean ± S.D. calculated from three independent and duplicate experiments with GAPDH used as the internal control are indicated by Columns, mean; bars, SD. (#) $p<0.05$ versus control (noninfected cells), p=not significant (ns), $p<0.01$ (**), $p<0.001$(***) and $p<0.0001$ (****), statistically significant compared with DV-infected untreated control. The statistics were performed by comparing results from treated to untreated DV infected cells utilizing ANOVA followed by Dunnett's Multiple Comparison Test.

**Table 4.8 Relative Quantitation (Fold of Induction) in MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA Expression of Vero Cells 48 hours post-treatment with ZOM at different Concentrations.**

| Target Gene | Treatment | Mean RQ Trial 1 | Mean RQ Trial 2 | Mean RQ Trial 3 | Mean RQ | SD | SEM |
|---|---|---|---|---|---|---|---|
| MMP-2 | DV-infected | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 | 0.00 |
| | DV+ZOM 6.25 µg/mL | 0.91 | 0.89 | 0.85 | 0.88 | 0.31 | 0.02 |
| | DV+ZOM 12.5 µg/mL | 0.64 | 0.71 | 0.67 | 0.67 | 0.04 | 0.020 |
| | DV+ZOM 25 µg/mL | 0.32 | 0.25 | 0.31 | 0.29 | 0.04 | 0.02 |
| | DV+ZOM 50 µg/mL | 0.05 | 0.12 | 0.23 | 0.13 | 0.09 | 0.05 |
| | DV+EGCG 100 µM | 0.87 | 0.75 | 0.84 | 0.82 | 0.06 | 0.04 |
| MMP-9 | DV-infected | 1 | 1 | 1 | 1.00 | 0.00 | 0.00 |
| | DV+ZOM 6.25 µg/mL | 0.88 | 0.76 | 0.78 | 0.81 | 0.06 | 0.04 |
| | DV+ZOM 12.5 µg/mL | 0.74 | 0.72 | 0.68 | 0.71 | 0.03 | 0.02 |
| | DV+ZOM 25 µg/mL | 0.51 | 0.48 | 0.43 | 0.47 | 0.04 | 0.02 |
| | DV+ZOM 50 µg/mL | 0.42 | 0.38 | 0.36 | 0.39 | 0.03 | 0.02 |
| | DV+EGCG 100 µM | 0.71 | 0.77 | 0.85 | 0.78 | 0.07 | 0.04 |
| TIMP-1 | DV-infected | 1 | 1 | 1 | 1.00 | 0.00 | 0.00 |
| | DV+ZOM 6.25 µg/mL | 1.19 | 1.28 | 1.3 | 1.26 | 0.06 | 0.03 |
| | DV+ZOM 12.5 µg/mL | 2.98 | 2.91 | 2.87 | 2.92 | 0.05 | 0.03 |
| | DV+ZOM 25 µg/mL | 3.12 | 3.02 | 3.23 | 3.12 | 0.11 | 0.06 |
| | DV+ZOM 50 µg/mL | 3.72 | 3.83 | 3.97 | 3.84 | 0.13 | 0.07 |
| | DV+EGCG 100 µM | 1.33 | 1.23 | 1.45 | 1.34 | 0.11 | 0.66 |
| TIMP-2 | DV-infected | 1 | 1 | 1 | 1.00 | 0.00 | 0.00 |
| | DV+ZOM 6.25 µg/mL | 2.73 | 2.67 | 2.34 | 2.58 | 0.21 | 0.12 |
| | DV+ZOM 12.5 µg/mL | 4.17 | 4.53 | 4.26 | 4.32 | 0.19 | 0.11 |
| | DV+ZOM 25 µg/mL | 5.88 | 5.56 | 5.86 | 5.77 | 0.18 | 0.10 |
| | DV+ZOM 50 µg/mL | 6.27 | 6.89 | 6.94 | 6.70 | 0.37 | 0.22 |
| | DV+EGCG 100 µM | 2.24 | 2.12 | 2.04 | 2.13 | 0.10 | 0.058 |

At a [6]-gingerol concentration of 6.25µg/mL no difference was observed in the MMP-2 and MMP-9 gene expression level. MMP-2 was reduced significantly to 0.85, 0.45, and 0.16 folds in response to [6]-gingerol treatment at concentrations of 12.5µg/mL, 25µg/ml and 50 µg/mL respectively (figure 4.17, A). The expression of MMP-9 was significantly down-regulated in response to [6]-gingerol treatment of 12.5µg/mL, 25 and 50µg/ml to 0.87, 0.76 and 0.68 respectively (figure 4.17, B).

Similarly, there was a significant increased in the expression level of the TIMP-1 gene in the [6]-gingerol treated DV infected cells with 6.25, 12.5, 25 and 50µg/ml to 1.28 ($p=0.0163$), 2.39 ($p<0.0001$), 3.01 ($p<0.0001$) and 3.51 folds respectively (figure 4.17, C). Moreover, expression of the TIMP-2 gene was significantly increased to 1.94, 4.05 and 5.64 folds in [6]-gingerol treated cells with 12.5, 25 and 50µg/ml concentration respectively and no significant difference observed in TIMP-2 gene expression at the lowest concentration of 6.25µg/ml (figure 4.17, D). EGCG upregulated the expression of TIMP-1 to 1.34 ($p=0.0046$) folds and TIMP-2 to 2.13 ($p=0.0074$) folds in comparison to DV infected cells.


MMP-2
Relative Quantitation (Fold of Induction)
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1.0
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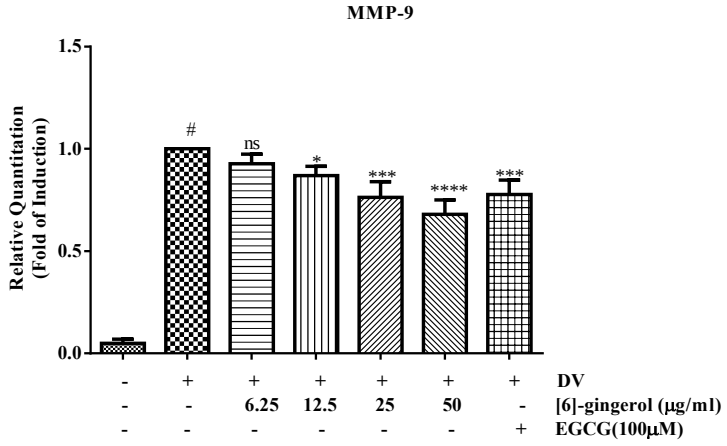
- + + + + + + DV
- - 6.25 12.5 25 50 - [6]-gingerol (µg/ml)
- - - - - - + EGCG(100µM)

A.


MMP-9
Relative Quantitation (Fold of Induction)
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1.0
0.5
0.0
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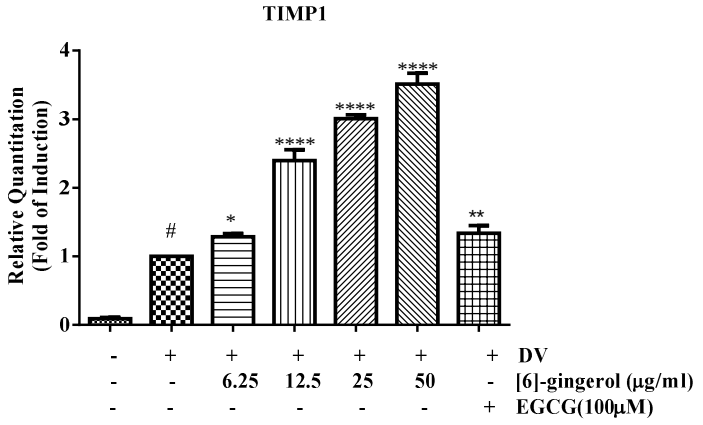
- + + + + + + DV
- - 6.25 12.5 25 50 - [6]-gingerol (µg/ml)
- - - - - - + EGCG(100µM)

B.


TIMP1
Relative Quantitation (Fold of Induction)
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- + + + + + + DV
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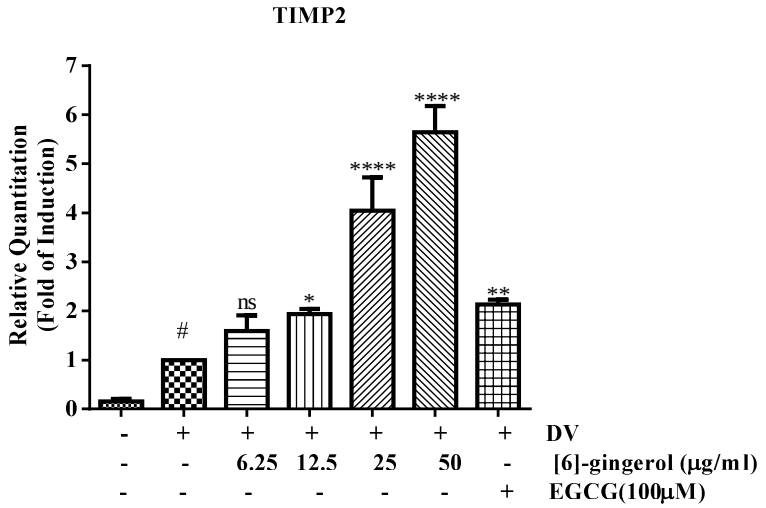
- - - - - - + EGCG(100µM)

C.




D.

**Fig 4.17 Concentration-dependent inhibitory effects of [6]-gingerol 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 to form a monolayer following infection with DV and treatment with various concentrations of [6]-gingerol and 100µM EGCG for 48 hours at 37°C. 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 and duplicate experiments with GAPDH used as the internal control are indicated by Columns, mean; bars, SD. (#) $p<0.05$ versus control (noninfected cells), p=not significant (ns), $p<0.05$ (*) $p<0.01$ (**), $p<0.001$(***) and $p<0.0001$ (****), statistically significant compared with DV-infected untreated control. The statistics were performed by comparing results from treated to untreated DV infected cells utilizing ANOVA followed by Dunnett's Multiple Comparison Test.

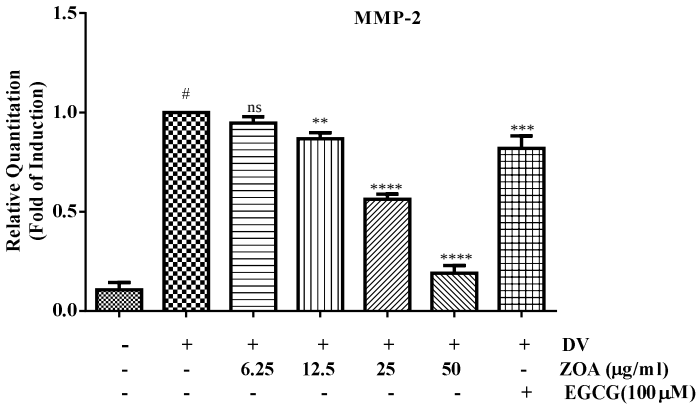
**Table 4.9 Relative Quantitation (Fold of Induction) in MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA Expression of Vero Cells 48 hours post-treatment with [6]-gingerol at different Concentrations.**

| Target Gene | Treatment | Mean RQ Trial 1 | Mean RQ Trial 2 | Mean RQ Trial 3 | Mean RQ | SD | SEM |
|---|---|---|---|---|---|---|---|
| MMP-2 | DV-infected-Untreated | 1 | 1 | 1 | 1 | 0.00 | 0.00 |
| | DV+[6]-gingerol6.25 µg/mL | 0.91 | 0.86 | 0.89 | 0.89 | 0.03 | 0.02 |
| | DV+[6]-gingerol 12.5 µg/mL | 0.84 | 0.79 | 0.91 | 0.85 | 0.66 | 0.04 |

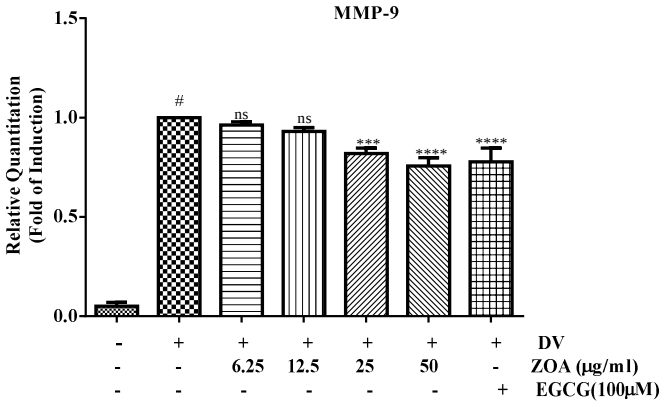
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | DV+[6]-gingerol 25 µg/mL | 0.42 | 0.39 | 0.54 | 0.45 | 0.08 | 0.05 |
| | DV+[6]-gingerol 50 µg/mL | 0.08 | 0.21 | 0.2 | 0.16 | 0.07 | 0.04 |
| | DV+EGCG 100 µM | 0.87 | 0.75 | 0.84 | 0.82 | 0.06 | 0.04 |
| MMP-9 | DV-infected | 1 | 1 | 1 | 1.00 | 0.00 | 0.00 |
| | DV+[6]-gingerol 6.25 µg/mL | 0.98 | 0.89 | 0.91 | 0.93 | 0.05 | 0.03 |
| | DV+[6]-gingerol 12.5 µg/mL | 0.92 | 0.86 | 0.83 | 0.87 | 0.05 | 0.03 |
| | DV+[6]-gingerol 25 µg/mL | 0.84 | 0.76 | 0.69 | 0.76 | 0.08 | 0.04 |
| | DV+[6]-gingerol 50 µg/mL | 0.75 | 0.68 | 0.61 | 0.68 | 0.07 | 0.04 |
| | DV+EGCG 100 µM | 0.71 | 0.77 | 0.85 | 0.78 | 0.07 | 0.04 |
| TIMP-1 | DV-infected | 1 | 1 | 1 | 1.00 | 0.00 | 0.00 |
| | DV+[6]-gingerol 6.25 µg/mL | 1.23 | 1.31 | 1.31 | 1.28 | 0.04 | 0.03 |
| | DV+[6]-gingerol 12.5 µg/mL | 2.21 | 2.48 | 2.49 | 2.39 | 0.16 | 0.09 |
| | DV+[6]-gingerol 25 µg/mL | 2.9 | 3.07 | 2.97 | 2.98 | 0.05 | 0.03 |
| | DV+[6]-gingerol 50 µg/mL | 3.67 | 3.51 | 3.35 | 3.51 | 0.16 | 0.09 |
| | DV+EGCG 100 µM | 1.33 | 1.23 | 1.45 | 1.34 | 0.11 | 0.06 |
| TIMP-2 | DV-infected | 1 | 1 | 1 | 1.00 | 0.00 | 0.00 |
| | DV+[6]-gingerol 6.25 µg/mL | 1.34 | 1.48 | 1.95 | 1.59 | 6.32 | 0.18 |
| | DV+[6]-gingerol 12.5 µg/mL | 1.83 | 1.96 | 2.03 | 1.94 | 0.10 | 0.06 |
| | DV+[6]-gingerol 25 µg/mL | 4.57 | 4.29 | 3.28 | 4.05 | 0.68 | 0.39 |
| | DV+[6]-gingerol 50 µg/mL | 5.02 | 5.89 | 6.01 | 5.64 | 0.54 | 0.31 |
| | DV+EGCG 100 µM | 2.24 | 2.12 | 2.04 | 2.13 | 0.10 | 0.06 |

At the ZOA concentration of 6.25µg/mL no difference was observed in the MMP-2 gene expression levels. MMP-2 gene expression 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 12.5µg/mL, 25µg/mL and 50µg/mL respectively (figure 4.18, A). At 6.25 and 12.5µg/mL ZOA no difference was observed in the MMP-9 expression level. The expression of MMP-9 was significantly down-regulated to 0.82 ($p=0.0001$) and 0.76 ($p<0.0001$) folds in response to ZOA treatment of 25 and 50µg/ml respectively (figure 4.18, B). 100µM of EGCG (Control) significantly down-regulated the expression of MMP-2 to 0.82 folds ($p=0.0002$) and MMP-9 to 0.78 folds ($p<0.0001$).

There was a significant increase in the expression level of the TIMP-1 gene in the ZOA treated DV infected cells with 12.5, 25 and 50µg/mL to 1.93-, 2.66- and 3.07-fold ($p<0.0001$) respectively (figure 4.18, C). Similarly expression of the TIMP-2 gene was increased to 1.92, 3.86 and 4.78 folds in ZOA-treated cells with 12.5, 25 and 50µg/ml respectively. No difference was observed at 6.25 µg/mL of ZOA treatment (figure 4.18, D). 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.




A.

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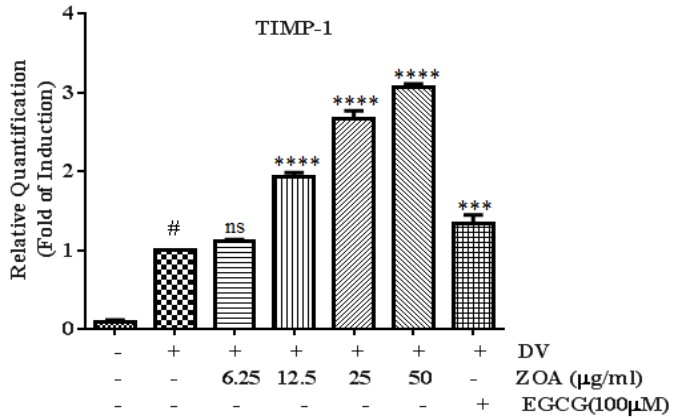
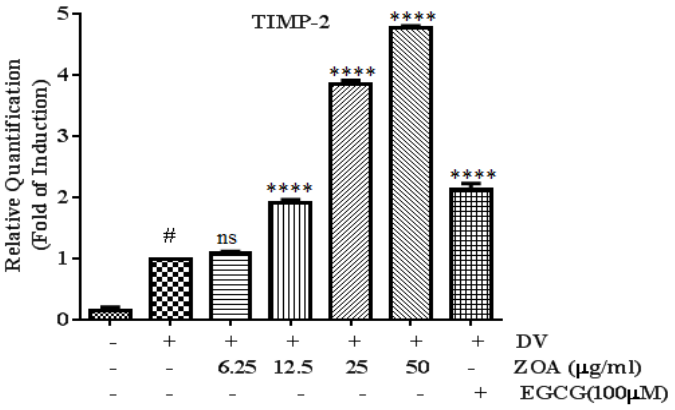



C.




D.

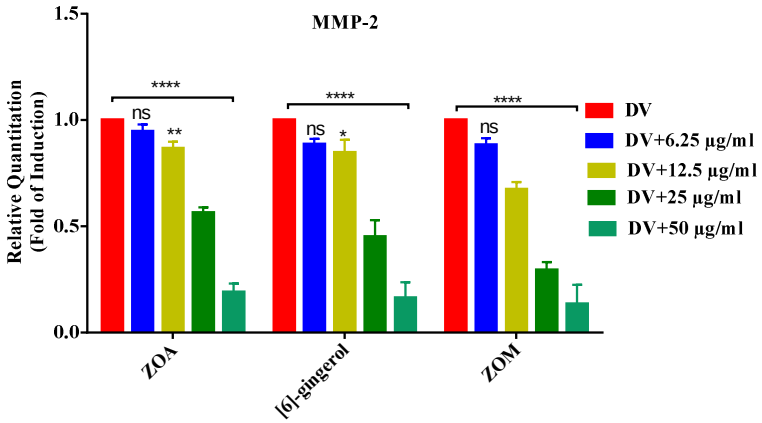
**Fig 4.18 Concentration-dependent inhibitory effects of ZOA on expression of MMPs and TIMPs in DV infected Vero cells.** A) MMP-2, B) MMP-9, C) TIMP-1, D) TIMP-2. Vero cells were plated at a density of 5x $10^4$ cells/mL with MEM supplemented with 2% FBS to form a monolayer following infection with DV and treatment with various concentrations of ZOA and 100µM EGCG for 48 hours at 37°C. 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 and duplicate experiments with GAPDH used as the internal control are indicated by Columns, mean; 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. The statistics were performed by comparing results from treated to untreated DV infected cells utilizing ANOVA followed by Dunnett's Multiple Comparison Test.

**Table 4.10 Relative Quantitation (Fold of Induction) in MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA Expression of Vero Cells 48 hours post-treatment with ZOA at different Concentrations.**

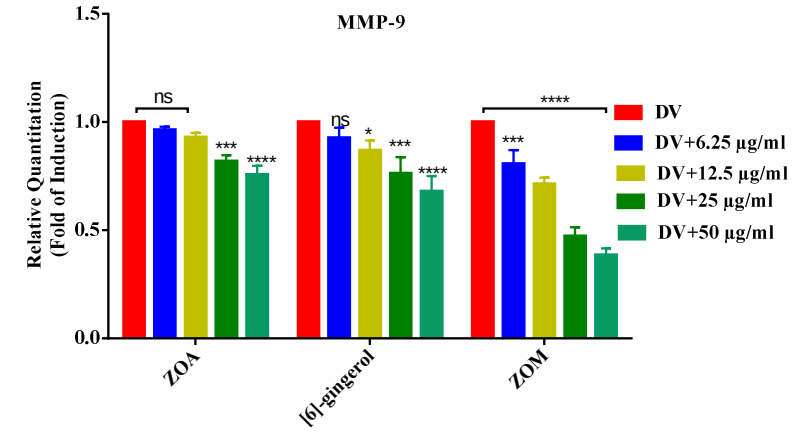
| Target Gene | Treatment | Mean RQ Trial 1 | Mean RQ Trial 2 | Mean RQ Trial 3 | Mean RQ | SD | SEM |
|---|---|---|---|---|---|---|---|
| MMP-2 | DV-infected | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 | 0.000 |
| | DV+ZOA 6.25 µg/mL | 0.91 | 0.97 | 0.96 | 0.95 | 0.03 | 0.019 |
| | DV+ZOA 12.5 µg/mL | 0.88 | 0.83 | 0.89 | 0.87 | 0.03 | 0.019 |
| | DV+ZOA 25 µg/mL | 0.56 | 0.59 | 0.54 | 0.56 | 0.02 | 0.015 |
| | DV+ZOA 50 µg/mL | 0.15 | 0.19 | 0.23 | 0.19 | 0.04 | 0.023 |
| | DV+EGCG 100 µM | 0.87 | 0.75 | 0.84 | 0.82 | 0.06 | 0.036 |
| MMP-9 | DV-infected | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 | 0.000 |
| | DV+ZOA 6.25 µg/mL | 0.96 | 0.98 | 0.95 | 0.96 | 0.01 | 0.008 |
| | DV+ZOA 12.5 µg/mL | 0.93 | 0.95 | 0.91 | 0.93 | 0.02. | 0..012 |
| | DV+ZOA 25 µg/mL | 0.84 | 0.79 | 0.83 | 0.82 | 0.02 | 0.015 |
| | DV+ZOA 50 µg/mL | 0.77 | 0.71 | 0.79 | 0.76 | 0.04 | 0.024 |
| | DV+EGCG 100 µM | 0.71 | 0.77 | 0.85 | 0.78 | 0.07 | 0.041 |
| TIMP-1 | DV-infected | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 | 0.000 |
| | DV+ZOA 6.25 µg/mL | 1.09 | 1.14 | 1.1 | 1.11 | 0.03 | 0.015 |
| | DV+ZOA 12.5 µg/mL | 1.98 | 1.87 | 1.94 | 1.93 | 0.06 | 0.032 |
| | DV+ZOA 25 µg/mL | 2.55 | 2.67 | 2.77 | 2.66 | 0.11 | 0.064 |
| | DV+ZOA 50 µg/mL | 3.02 | 3.11 | 3.07 | 3.07 | 0.05 | 0.026 |
| | DV+EGCG 100 µM | 1.33 | 1.23 | 1.45 | 1.34 | 011 | 0.064 |
| TIMP-2 | DV-infected | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 | 0.000 |
| | DV+ZOA 6.25 µg/mL | 1.1 | 1.07 | 1.12 | 1.10 | 0.03 | 0.015 |
| | DV+ZOA 12.5 µg/mL | 1.88 | 1.97 | 1.92 | 1.92 | 0.05 | 0.026 |
| | DV+ZOA 25 µg/mL | 3.86 | 3.92 | 3.81 | 3.86 | 0.06 | 0.032 |
| | DV+ZOA 50 µg/mL | 4.81 | 4.75 | 4.78 | 4.78 | 0.03 | 0.017 |
| | DV+EGCG 100 µM | 2.24 | 2.12 | 2.04 | 2.13 | 0.10 | 0.058 |

The expression of MMP-2 and MMP-9 in DV-infected Vero cells was decreased by ZOM, pure [6]-gingerol and ZOA treatment in a dose-dependent manner (Table 4.9, 4.10 and 4.11). Results from this study showed that there is a significant difference in the mean percentage downregulation of MMP-2 and

MMP-9 expression by ZOM, pure [6]-gingerol and ZOA ($p<0.0001$) at their higher concentrations, in which Tukey's HSD post hoc analysis indicated that ZOM exerts the highest mean percentage downregulation of MMP-2 and MMP-9 expression, followed by [6]-gingerol, while the level of expression from ZOA treatment do not differ significantly at its lower concentration. The effect of ZOM, pure [6]-gingerol and ZOA exert a concentration dependent downregulation of MMP-2 and MMP-9 expression. Similarly ZOM exerted the highest mean percentage upregulation of mRNA expression of TIMP-2 and TIMP-1, followed by [6]-gingerol and ZOA at their higher concentrations, while no significant difference was exerted in their mRNA expressions at lower concentrations of extracts and pure [6]-gingerol.




A.

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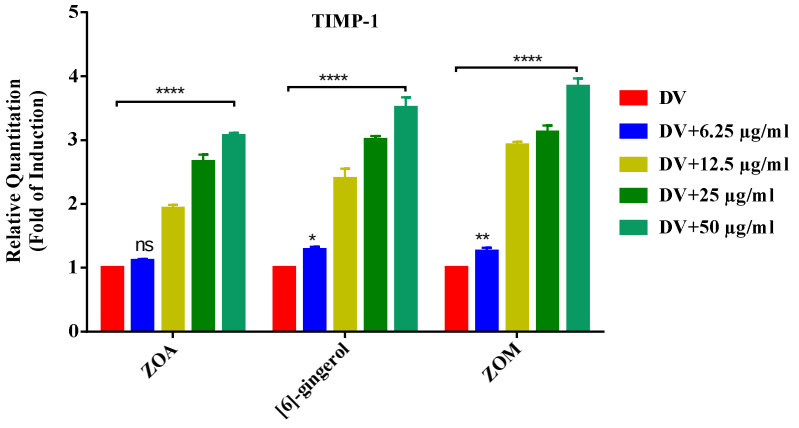


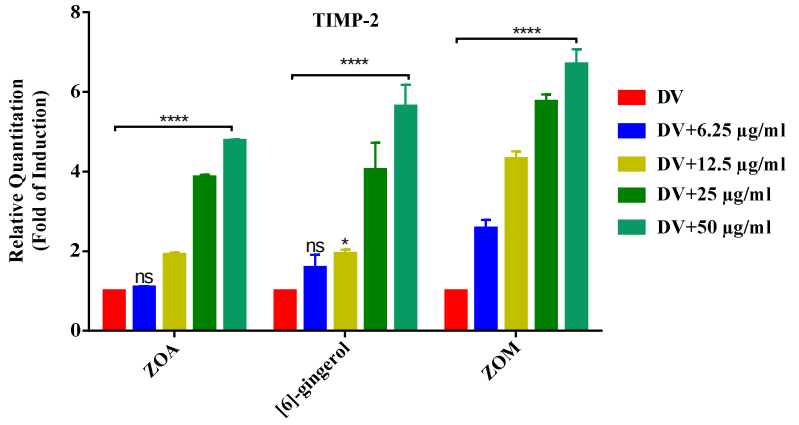


**Fig 4.19 Effects of ZOA, [6]-gingerol and ZOM on the mRNA expression of DV infected (m.o.i=1ffu/cell) Vero cells. MMP-2 (A), MMP-9 (B), TIMP-1 (C) and**

**TIMP-2 (D).** Dose dependent inhibition of MMP-2 expression after treatment with ZOA, [6]-gingerol and ZOM as demonstrated by real time RT-PCR. Each bar represents the mean ± S.D. calculated from three independent and duplicate experiments with GAPDH used as the internal control are indicated by Columns, mean; bars, SD. (#) p=not significant (ns), p<0.05 (*), p<0.01 (**), p<0.001(***) and p<0.0001 (****), statistically significant compared with DV-infected untreated control.

**4.9 [6]-gingerol modulates mRNA expressions of MMP-2, MMP-9, TIMP-1 and TIMP-2 in DV infected cells in a concentration-and time-dependent manner.**

**4.9.1 MMP-2, MMP-9, TIMP-1 and TIMP-2 are overexpressed in DV-infected Vero cells**

According to the results shown in figure 4.20, 4.21, 4.22and 4.23 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 mRNAs were overexpressed in Vero cells upon DV infection. MMP-2 was upregulated to 8.74, 9.59 and 9.71folds (p<0.0001) and MMP-9 was upregulated to 5.03, 5.85 and 6.03 folds (p<0.0001) incomparison 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 mRNA to 1.94, 2.06 and 2.70 folds (p<0.0001) and TIMP-2 mRNA to 2.36, 3.00 and3.23 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 increased production of natural tissue inhibitors MMP-9 and MMP-2, respectively.

**4.9.2 [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 a decrease in DV-induced MMP-2 mRNA expression in a dose and time-dependent fashion (figure 4.20). At the [6]-gingerol concentration of 6.25µg/mL no difference observed in the MMP-2 gene expression compared to the untreated DV infected cell. MMP-2 mRNA 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 25µg/ml and 50µg/ml respectively after 24 hrs of incubation. The expression of MMP-2 was reduced significantly to 9.19 folds ($p=0.0120$) at 12.5µg/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 12.5µg/ml, 3.76 folds ($p=<0.0001$) at 25µg/mL and 2.42 folds ($p=<0.0001$) at 50µg/ml in response to [6]-gingerol trearment after 72 hrs of incubation.

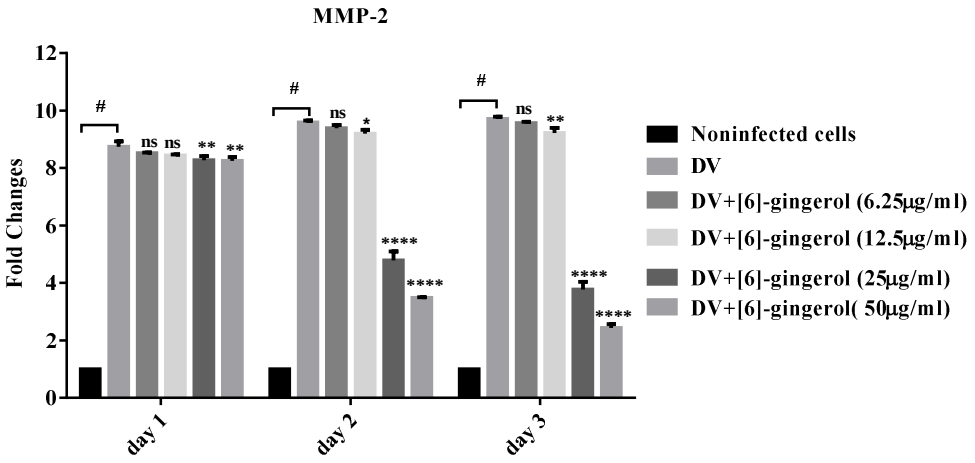


**Fig 4.20 Expression of MMP-2 in non-infected, DV-infected and [6]-gingerol-treated DV infected Vero cells.** For quantitative analysis, total RNA was isolated from non-infected, DV-infected and DV-infected cells after [6]-gingerol-treatment (6.25, 12.5, 25 and 50 µg/ml for 24, 48 and 72 hrs of incubation). 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; 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.

### 4.9.3 [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 expressionlevel at either treatment concentration for 24hrs of incubation and at concentration of 6.25 and 12.5µg/ml for48 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 25µg/ml and 50µg/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 50µg/ml respectively after72 hrs of incubation (figure 4.21). These results suggest that [6]-gingerol may induce anti-MMPs effect in both a time-and-concentration-dependent manner in DV infected cells.

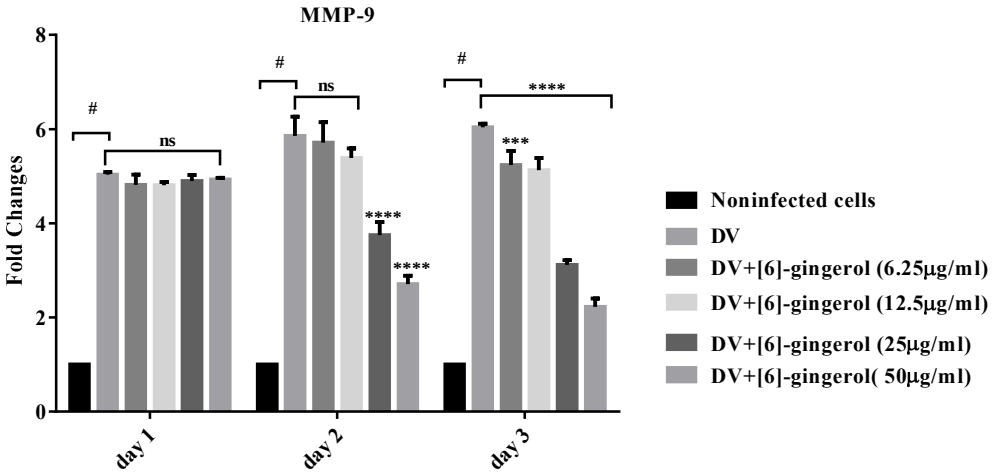


**Fig 4.21 Expression of MMP-9 in non-infected, DV-infected and [6]-gingerol-treated DV infected Vero cells.** For quantitative analysis, total RNA was isolated from non-infected, DV-infected and DV-infected cells after [6]-gingerol-treatment (6.25, 12.5, 25 and 50µg/ml for 24, 48 and 72 hrs of incubation). 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; 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.

### 4.9.4 [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 concentrations of 6.25, 12.5, 25 and 50µg/mL compared to

the untreated DV infected cells. There was no significant difference observed in the TIMP-1 mRNA expression at a concentration of 6.25µg/mL 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 mRNA was significantly increased to 2.52 folds ($p=0.0001$), 2.63 folds ($p<0.0001$) and 2.92 folds ($p<0.0001$) respectively compared to the untreated DV-infected cells. After 72 hrs incubation, there was a significant increase 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 50µg/ml respectively (figure 4.22).

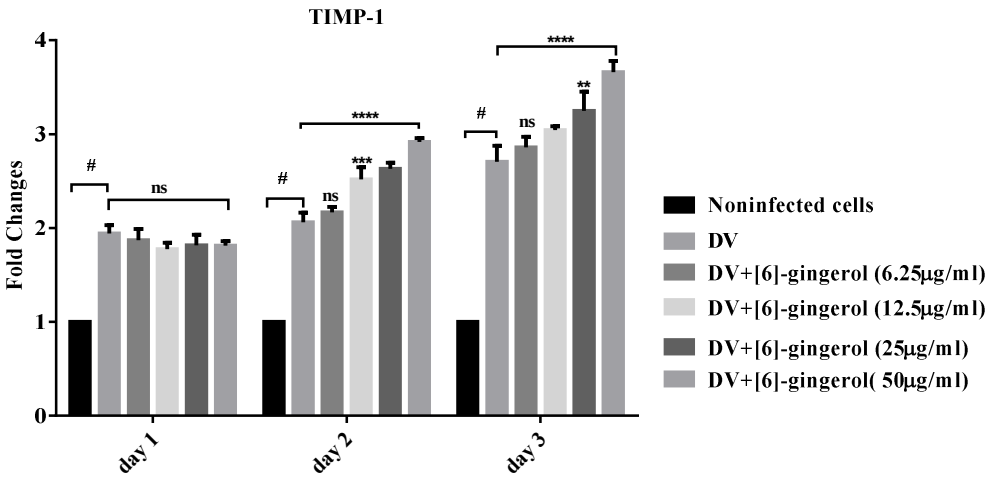


**Fig 4.22 Expression of MMP-2 in non-infected, DV-infected and [6]-gingerol-treated DV infected Vero cells.** For quantitative analysis, total RNA was isolated from non-infected, DV-infected and DV-infected cells after [6]-gingerol-treatment (6.25, 12.5, 25 and 50µg/ml for 24, 48 and 72 hrs of incubation). 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; bars, SD.

p<0.0001 (#), statistically significant compared with non-infected cells; p = notsignificant (ns), p<0.01 (**), p<0.001 (***), and p<0.0001 (****), statistically significant comparedwith DV-infected untreated control.

**4.9.5 [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 [6]-gingerol concentration of 6.25 and 12.5µg/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/mlrespectively 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 50µg/ml after 48 hrs of incubation respectively (figure 6). After 72 hrs incubation, there was a significant increase in the mRNA expression level of 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-infectedcells with 6.25µg/ml, 12.5µg/ml, 25 and 50µg/ml respectively (figure 4.23). The effect of higher concentrations of [6]-gingerol 25 and 50 µg/ml after 48 hrs of treatment, suggest that [6]-gingerol induces an anti-MMP effect in a time and-concentration-dependent manner in DV infected cells.

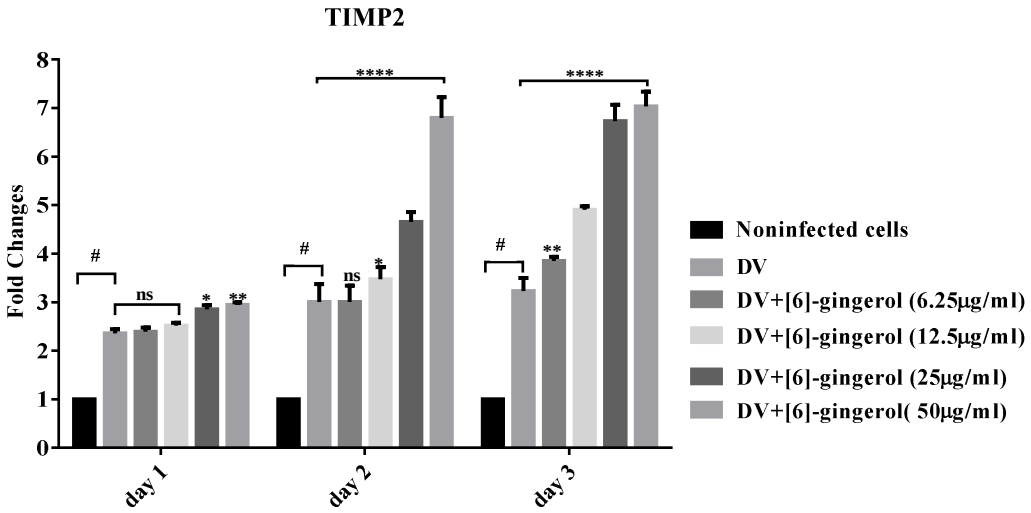


**Fig 4.23 Expression of MMP-2 in non-infected, DV-infected and [6]-gingerol-treated DV infected Vero cells.** For quantitative analysis, total RNA was isolated from non-infected, DV-infected and DV-infected cells after [6]-gingerol-treatment (6.25, 12.5, 25 and 50µg/ml for 24, 48 and 72 hrs of incubation). 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.

*Zingiber officinale* rhizome extracts and its biologically active compounds, including [6]-gingerol and shogaols were reported to be effective using *in vitro* models of various disease conditions by modulating the MMP-2 and MMP-9 gene expression, protein expression and secretion. However, the efficacy of pure [6]-gingerol and ginger extracts in modulating MMPs and TIMPs in DHF/DSS were not explored. Here, we examined the effects of ZOA, ZOM and pure [6]-gingerol on MMP-2,MMP-9 and TIMP-1, TIMP-2 expression using reverse transcription using real-time RTPCR and found that ZOM, pure [6]-gingerol and

ZOA significantly downregulated the mRNA expression of MMP-2 and MMP-9, whereas there was an upregulation in the expression of TIMP-1 and TIMP-2 in DV-infected cells at higher concentrations, suggesting that *Z. officinale* rhizome extracts and pure [6]-gingerol induce anti-MMP effect in a concentration-dependent manner in DV-infected cells. ZOA, ZOM and pure [6]-gingerol may also affect matrixmetalloproteinases expression indirectly via their action on transcriptional factors like AP-1 and NF-kB, which regulate transcription of MMPs (Folgueras *et al.*, 2004).

Ling *et al*., (2008) showed that [6]-Shogaol displayed the greatest anti-invasive effect in association with a dose-dependent reduction in MMP-9 gene expression, protein expression and secretion (Ling *et al*., 2010). The molecular mechanism involved in the anti-invasive activity of shogaols on MDA-MB-231 breast cancer cells involves the down-regulation of MMP-9 transcription by targeting the NFκB activation cascade (Ling *et al*., 2010).

Weng *et al*., (2010) 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. MMP-9 activity was decreased by [6]-gingerol and [6]-shogaol treatment through the enhanced protein level of TIMP-1 protein and reducing the uPA activity which might contribute to the anti-invasion activity.

Lee *et al*., (2008) also demonstrated that [6]-gingerol inhibited metastasis in MDA-MB-2 cells through the dose dependent inhibition of MMP-2 and MMP-9. Yanti (2011) showed that selected *Zingiber officinale* rhizome extracts (5 µg per mL), reduced the MMP-9 secretion, gene and protein expression, secretion, in an LPS-induced *in vitro* model of artherosclerosis.

These reports support our findings that the natural compounds present in ZOM, ZOA and [6]-gingerol can play an important anti-MMPs role. Our study is the first of its kindto show that ZOM, pure [6]-gingerol and ZOA 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 concentration-dependent manner. EGCG, used as control an anti-MMP agent, was found to downregulate the mRNA expressions of MMP-2, MMP-9 and upregulate the mRNA expressions of TIMP-1 and TIMP-2. These findings are also supported by several studies in the literture. Such as, in neuroblastoma, fibrosarcoma, glioblastoma, prostate cancer, and human gastric cancercells, EGCG inhibited MMP-2 and MMP-9 while inducing the activity of their inhibitors TIMP-1 and TIMP-2 (Maeda-Yamamoto *et al*., 1999; Ramos, 2007; Yang *et al*., 2010; Slivova *et al*., 2005). Similarly, in human breast cancer cells, EGCG treatment reduced MMP-2 activity and the expression of focal adhesion kinase (FAK), membrane type-1-MMP (MT1-MMP), nuclear factor (NF)-kappaB, VEGF, and the adhesion of cells to the extracellular matrix

(ECM) (Sen*et al*., 2009). Likewise, in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, green tea polyphenol infusion resulted in marked inhibition of effectors of angiogenesis and metastasis, notably VEGF, uPA, MMP-2, and MMP-9 (Khan *et al*., 2009).

All MMPs are synthesized as preproenzymes with an endoplasmic reticulum targeting signal peptide and secreted as inactive zymogens or active enzymes (Dzwonek *et al*., 2004). Once secreted, MMPs are activated via cleavage of the propeptide domain, removing the inhibiting cysteine residue from the catalytic cleft (Nagase and Woessner, 1999). This cleavage can be mediated enzymatically by other MMPs or proteases such as plasmin (Candelario-Jalil *et al.*, 2009). Activation can also be mediated non-enzymatically, which involves disruption of the inhibitory interaction between the $Zn^{2+}$ residue and the cysteine residue. Catalytic MMP activation can also be mediated by other MMPs. For example, MT1-MMP, MT2-MMP and MT5-MMP have been shown to activate proMMP-2 (Kinoh *et al*., 1996; Llano *et al*., 1999; Morrison *et al*., 2001). As well, MMP-2, MMP-3 and MMP-13 are able to cleave and activate proMMP-9 (Fridman *et al*., 1995; Ogata *et al*., 1992; Knauper *et al*., 1997). ProMMP-2 can be activated by MT1-MMP, but this activation is tightly regulated by the complexing of tissue inhibitor of metalloproteinase (TIMP)-2 and MMP- 2 in cells (Deryugina *et al*., 2001). It is thought that the TIMP-2 subunit is required for the localization of the whole complex and thus, MMP-2 localization to the cell membrane where

it can then be proteolytically activated by MT1-MMP (Emmert-Buck *et al*., 1995). Thus, MMPs play a significant role in the modulation of each other and allow for even tighter regulation of activity. Once they are activated however, the proteolytic activity of MMPs is modulated either by nonspecific protease inhibitors, α2-macroglobulin or by a family of endogenous regulators such as tissue inhibitor of metalloproteinases (TIMPs). There are 4 known members of the TIMP family: TIMP-1, -2, -3 and -4, ranging from 21-30 kDa in size. TIMPs noncovalently bind to and inhibit MMPs in a 1:1 stoichiometric ratio (Murphy, 2011). The mechanism by which TIMPs inhibit the catalytic activity of MMPs is not well known. Studies have reported on the interaction between TIMP-1 and MMP-3: TIMP-1 possesses cysteine residue that is capable of chelating the catalytic Zn2+, thus disrupting its ability to carry out hydrolytic attack on other species. TIMP-1 exhibits a preference for MMP-9 and TIMP-2 for MMP-2 respectively (Goldberg *et al*., 1992). TIMP-1 shows a lower affinity for the membrane-type MMPs (Nagase and Murphy, 2008). Additionally, TIMP-2 and TIMP-3 show a lower affinity for MMP-3 and MMP-7 than TIMP-1 (Hamze*et al*., 2007). TIMP-3 displays a wider range of MMP inhibition than the other TIMPs. The expression of TIMP-1 and TIMP-3 are thought to be highly inducible, whereas TIMP-2 expression is mostly constitutive (Verstappen and Von den Hoff, 2006). The major targets of the MMPs are the proteins that compose the extracellular matrix and basal lamina such as fibronectin, gelatin, collagen and

proteoglycans; indeed, virtually all protein components of the extracellular matrix (ECM) can be degraded by the MMPs (Dzwonek*et al*., 2004). They serve to modulate tissue architecture through turnover of the ECM and intercellular junctions.

**4.10 Permeability assay**

**4.10.1 Conditioned Media (CM) from DV-infected cells increase cell monolayer permeability**

DV infection has been shown to induce the production of soluble factors that could indirectly enhance vascular leakage. The molecules MMP-2 and MMP-9 have been reported to play an essential role in increased in vascular endothelial permeability (Luplerdlop*et al*., 2006; Luplerdlop and Misse, 2008). To evaluate the possible role for soluble factors in permeability, the capacity of conditioned media (CM) collected from DV infected Vero cells to modify cell monolayer permeability was measured as the amount of FITC-conjugated dextran that could be passed through a confluent monolayer of Madin-Darby Canine Kidney Epithelial Cells (MDCK) seeded onto transwell plates. The same concentrations of CM that were subjected to zymography and showed increased activities of MMP-2 and MMP-9 following upon DV infection were utilized in the permeability assays. Following exposure of confluent MDCK to CM from DV infected Vero cells for 24h, the ability of FITC-conjugated dextran to pass from

the upper chamber to the lower chamber of the transwell system suggests that CM from DV-infected Vero cells contain soluble and active factors which account for the increase of the MDCK monolayer permeability up to 70.73% ($p<0.0001$) compared to treatment with CM collected from non-infected cells. Indeed, CM collected from DV infected Vero cells increased MDCK cell monolayer permeability in a MMPs-dependent fashion as indicated by reduction in the permeability of MDCK monolayer to 13.97% ($p=0.0003$) in the presence of 10mMol EDTA (metalliproteinases inhibitor), which is similar to the observed basal level (figure 4.24) (Appendix M.14.A).

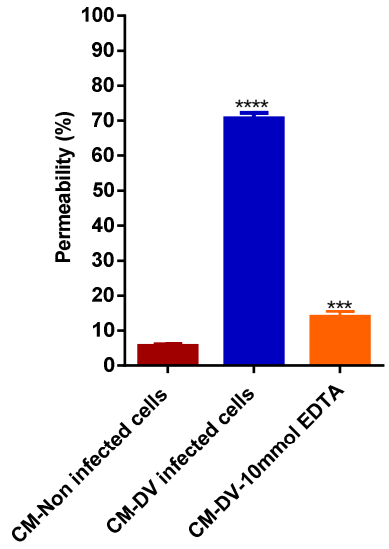


**Fig 4.24 Relative permeability induced by CM from DV infected cells.** CM collected from DV infected Vero cells increased MDCK cellmonolayer permeability in a MMPs-dependent fashion. MDCK cells were grown on 24-well transwell polyethylene membranes were exposed to CM collected from DV3 infected Vero cells and incubated for 1 hour at 37°C in the presence and absence of 10mMol EDTA (metalloproteinases inhibitor). Subsequently, these untreated and treated CM were added to confluent MDCK cells on 24-well transwell

polyethylene membranes for 24h. After the addition of fluorescein isothiocyanate-dextan to the MDCK monolayer, the extent of permeability was determined by measuring fluorescence in the lower transwell by spectrofluorometry. Data (mean ± SD) are representative of three independent and duplicate experiments; (***) p<0.001, ($^{**}$) p< 0.01 vs controls (CM from non-infected cells).

**4.10.2 Treatment of Conditioned Media (CM) from DV-infected cells with ZOM, [6]-gingerol and ZOA decrease cell monolayer permeability**

Various concentrations of ginger extracts (ZOA, ZOM), [6]-gingerol, and 100 µM of the control EGCG, were tested for their ability to inhibit permeability induced by the CM collected from the DV-infected Vero cells. The MDCK monolayers were incubated with CM from DV-infected Vero cells for 24 h in presence and absence of various concentrations of test compounds. As shown in Figure 6, ZOM [6]-gingerol significantly inhibit the permeability in dose dependent manner as compared to the CM from DV infected cells alone.

There was a significant reduction in the relative permeability of MDCK monolayer after incubation with CM in presence of ZOM of concentrations 6.25, 12.5, 25 and 50µg/ml to 86.80% (p=0.0152), 81.39% (p<0.0001), 43.06% (p<0.0001) and 7.32% (p<0.0001) respectively (figure 4.25). Indeed ZOM at 50µg/ml reduced the permeability of the MDCK monolayer similar to basal level.

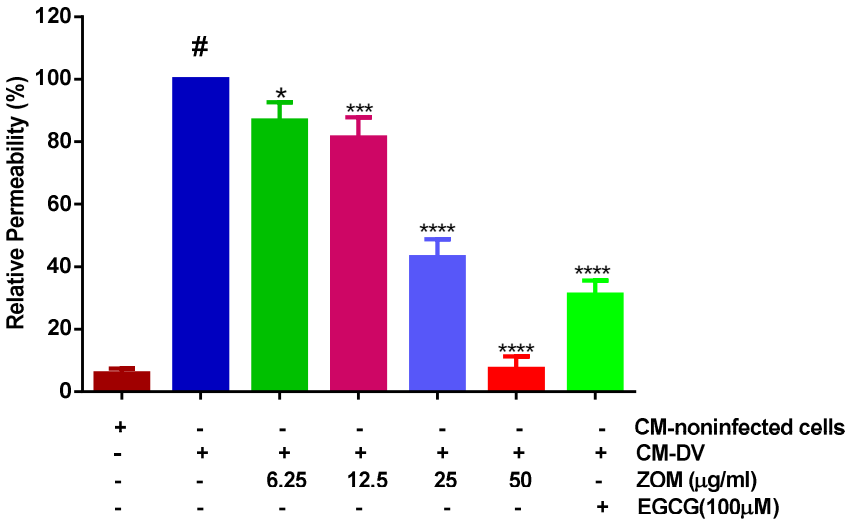


**Fig 4.25 Relative permeability induced by CM collected from DV-infected Vero cells following treatment wit ZOM.** MDCK cells grown on 24-well transwell polyethylene membranes were exposed to CM collected from DV3 infected Vero cells incubated for 1 hour at 37°C in the presence and absence of different concentrations of ZOM (6.25µg-50µg/ml), EGCG (100µM) and mock control (CM from uninfected Vero cells). Subsequently, these control, untreated and treated CM were added to confluent MDCK cells on 24-well transwell polyethylene membranes for 24 h at 37°C in 5% CO2. Following incubation, FITC-conjugated Dextran was added within the top chamber and permeability was measured by reading in the bottom chamber containing the infiltrated FITC-dextran at an excitation wavelength of 485 nm and an emission of 530 nm by spectrofluorometry. Columns show means of three independent and duplicate experiments and bars, SD; (#) $p<0.05$ versus control (CM-noninfected cells); p = not significant (ns), $p<0.01$ (**) and $p<0.0001$ (****) versus DV-infected group. The statistics were performed by comparing results from treated CM to untreated DV infected CM control utilizing ANOVA followed by Dunnett's Multiple Comparison Test.

Similarly relative permeability was significantly decreased to 82.97% ($p=0.0014$), 57.32 ($p<0.0001$), 32.88% ($p<0.0001$) when treated with [6]-gingerol at a concentration of 12.5, 25 and 50 µg/ml respectively and no significant difference was observed in the relative permeability at its low concentration of

6.25µg/ml (figure 4.26). [6]-gingerol at50µg/ml reduced the permeability of MDCK similar to those observed with 100 µM EGCG, a known potent MMP inhibitor.

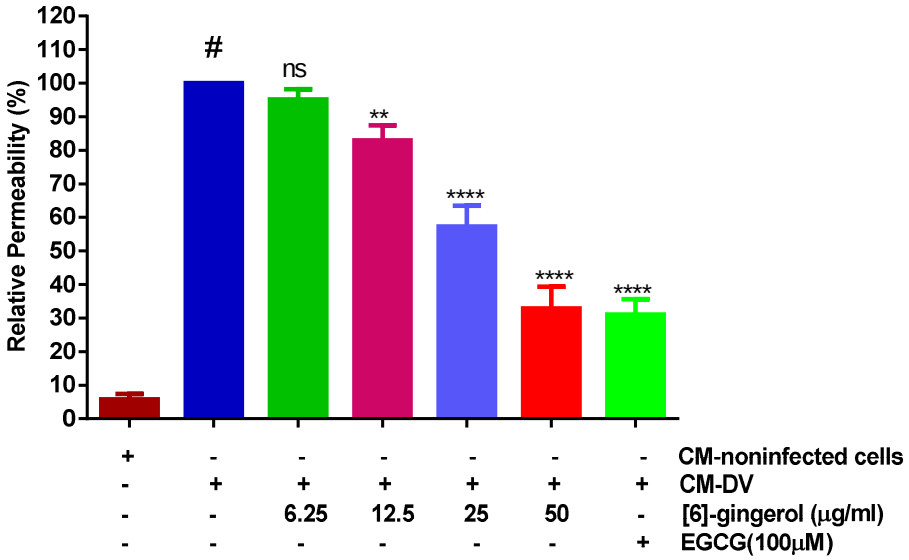


**Fig 4.26 Relative permeability induced by CM collected from DV-infected Vero cells following treatment with [6]-gingerol.** MDCK cells grown on 24-well transwell polyethylene membranes were exposed to CM collected from DV3 infected Vero cells and incubated for 1 hour at 37°C in the presence and absence of different concentrations of [6]-gingerol (6.25µg-50µg/ml), EGCG (100µM) and mock control (CM from uninfected Vero cells). Subsequently, these control, untreated and treated CM were added to confluent MDCK cells on 24-well transwell polyethylene membranes for 24 h at 37°C in 5% CO2. Following incubation, FITC-conjugated Dextran was added within the top chamber and permeability was measured by reading in the bottom chamber containing the infiltrated FITC-dextran at an excitation wavelength of 485 nm and an emission of 530 nm by spectrofluorometry. Columns show means of three independent and duplicate experiments and bars, SD; (#) $p<0.05$ versus control (CM-noninfected cells); p = not significant (ns), $p<0.01$ (**) and $p<0.0001$ (****) versus DV-infected group. The statistics were performed by comparing results from treated CM to untreated DV infected CM control utilizing ANOVA followed by Dunnett's Multiple Comparison Test.

ZOA reduced the relative permeability to 81.84% (p=0.0036), 63.93% (p<0.0001) at concentrations of 25 and 50 µg/ml respectively. There was no significant reduction in the permeability of MDCK monolayer exposed to CM with lower concentrations of ZOA. EGCG significantly reduced the permeability to 31.11% (p<0.0001) compared to non treated control (CM from DV infected cells without treatment). These results highlight the important capability of ZOM, [6]-gingerol and ZOA to reduce permeability, induced by CM collected from DV infected Vero cells which has been shown to contain secreted MMPs.

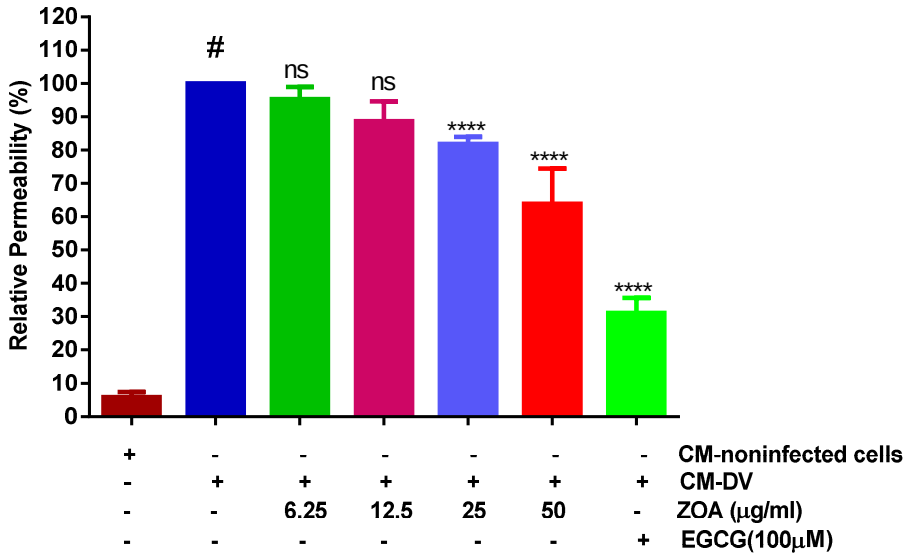


**Fig 4.27 Relative permeability induced by CM collected from DV-infected Vero cells following treatment with ZOA.** MDCK cells grown on 24-well transwell polyethylene membranes were exposed to CM collected from DV3 infected Vero cells and incubated for 1 hour at 37°C in the presence and absence of different concentrations of ZOA (6.25µg-50µg/ml), EGCG (100µM) and mock control (CM from uninfected Vero cells). Subsequently, these control, untreated and treated CM were added to confluent MDCK cells on 24-well transwell polyethylene membranes for 24 h at 37°C in 5% CO2. Following incubation, FITC-conjugated

Dextran was added within the top chamber and permeability was measured by reading in the bottom chamber containing the infiltrated FITC-dextran at an excitation wavelength of 485 nm and an emission of 530 nm by spectrofluorometry. Columns show means of three independent and duplicate experiments and bars, SD; (#) $p<0.05$ versus control (CM-noninfected cells); p = not significant (ns), $p<0.01$ (**) and $p<0.0001$ (****) versus DV-infected group. The statistics were performed by comparing results from treated CM to untreated DV infected CM control utilizing ANOVA followed by Dunnett's Multiple Comparison Test.

**Table 4.11 Relative Permeability of MDCK monolayers with CM collected from DV-infected Vero Cells 24 hours post-treatment with ZOM, ZOA and [6]-gingerol at different concentrations.**

| Treatment | Mean Relative Permeability (%) | SD | SEM |
|---|---|---|---|
| CM-DV (Untreated) | 100 | 0.0 | 0.0 |
| CM-DV+ZOM 6.25 µg/mL | 87 | 5.8 | 3.4 |
| CM-DV+ZOM 12.5 µg/mL | 81 | 6.4 | 3.7 |
| CM-DV+ZOM 25 µg/mL | 43 | 5.8 | 3.3 |
| CM-DV+ZOM 50 µg/mL | 7.3 | 3.9 | 2.3 |
| CM-DV+EGCG 100 µM | 31 | 4.5 | 2.6 |
| CM-DV (Untreated) | 100 | 0.0 | 0.0 |
| CM-DV+[6]-gingerol 6.25 µg/mL | 95 | 3.0 | 1.7 |
| CM-DV+[6]-gingerol 12.5 µg/mL | 83 | 4.4 | 2.6 |
| CM-DV+[6]-gingerol 25 µg/mL | 57 | 6.1 | 3.6 |
| CM-DV+[6]-gingerol 50 µg/mL | 33 | 6.5 | 3.7 |
| CM-DV+EGCG 100 µM | 31 | 4.5 | 2.6 |
| CM-DV (Untreated) | 100 | 0.0 | 0.0 |
| CM-DV+ZOA 6.25 µg/mL | 95 | 3.7 | 2.1 |
| CM-DV+ZOA 12.5 µg/mL | 89 | 5.9 | 3.4 |
| CM-DV+ZOA 25 µg/mL | 82 | 2.2 | 1.3 |
| CM-DV+ZOA 50 µg/mL | 64 | 1.1 | 6.1 |
| CM-DV+EGCG 100 µM | 31 | 4.5 | 2.6 |

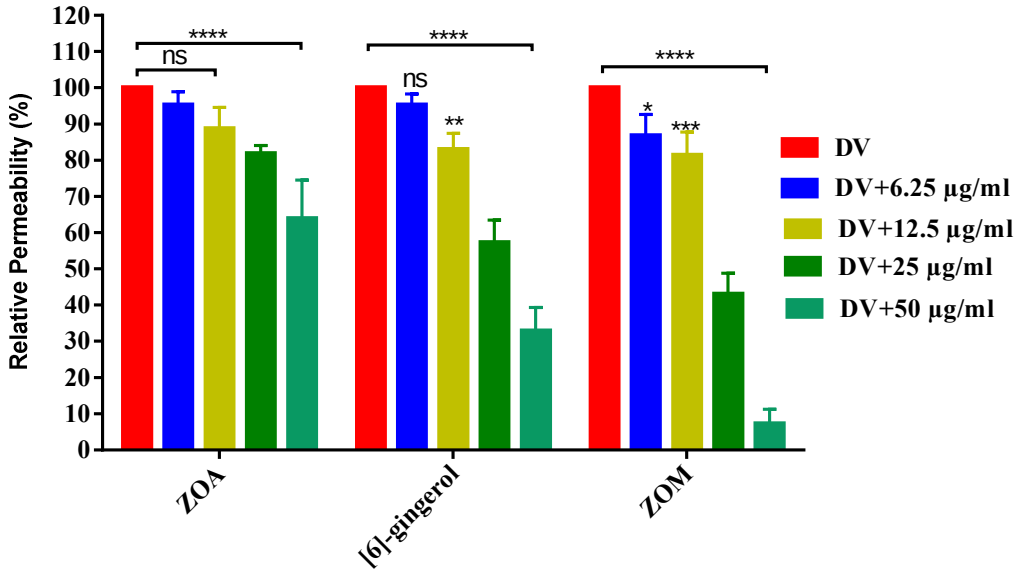


**Fig 4.28 Relative permeability induced by CM collected from DV-infected Vero cells following treatment with ZOA, [6]-gingerol and ZOM.** Columns show means of three independent and duplicate experiments and bars, SD; (*) $p<0.05$ versus control (CM-noninfected cells); p = not significant (ns), $p<0.01$ (**) and $p<0.0001$ (****) versus DV-infected group.

Severe dengue is characterized by hemorrhage and vascular permeability indicating the involvement of endothelial cells in pathogenesis. The interplay between endothelial cells (ECs) and dengue virus pathogenesis is well known. In this study, we have shown increased expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in DV infected Vero cells and increased activity of MMP-2 and MMP-9 in the conditioned media collected from DV-infected Vero cells leading to increase in MDCK monolayer permeability which is in agreement with the observations of Luplerdlop *et al.*, (2006). Those authors 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. They had demonstrated the molecular mechanism of MMPs in increased vascular permeability that relates with a loss of expression of PECAM-1 and vascular endothelium-cadherin cell adhesion molecules and redistribution of F-actin fibres. Indeed, these *in vitro* observations have been confirmed in an *in vivo* vascular leakage mouse model by Luplerdlop *et al*., (2006). Similarly, in another study conducted by Luplerdlop and Misse, they demonstrated that DV-induced MMP-2 overproduced by direct infection of endothelial cells may contribute to the pathogenesis of severe dengue infection. They have shown that infection of primary human macrovascular endothelial cells (MVEC) by DV results in overproduction of MMP-2 and to a lesser extent of MMP-9, leading to enhanced endothelial permeability which was associated with loss of expression of the vascular endothelium-cadherin cell-cell adhesion (Luplerdlop and Misse, 2008). Misse *et al*., 2001, found infection with that another virus, human immunodeficiency virus (HIV) infection enhanced the level of MMP-2 and MMP-9 leading to change in blood vessel permeability.

Moreover, our data are supported by findings of other clinical studies as well. Kubelka *et al*., (2010) observed the increased levels of circulating MMP-9, MMP-12 and MMP-13 in DF patients and found a higher amount of MMP-9 in

patients with the severe form of the illness rather than with the milder variety. Similarly in another study conducted by Waidab *et al*., (2008) also demonstrated significant elevation of MMP-9mRNA expression in children with DHF suggesting the role of this mediator in the pathogenesis. 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.

In this study, we present the first evidence to our knowledge that CM from DV-infected cells induced permeability which was markedly reduced in dose dependent manner by ZOM, pure [6]-gingerol and ZOA treatment. This reduction in the permeability might be due to depletion of the MMPs expression and activities and increased expression of TIMP-1 and TIMP-2 following treatment with ZOM, [6]-gingerol and ZOA as demonstrated by zymography and quantitative real time RT-PCR. All these results indicate that the stronger anti-MMPs activities of ZOM may be partially due to the higher content of total phenolics and [6]-gingerol. The activity may also be due to some other structural characteristics, such as phytochemicals composition. Together these are considered as main factors influencing anti-MMP-2,-9 activities and modulatory effects on the expressions of MMP-2, -9 and TIMP-1,-2 leading to decrease in permeability induced by DV-infection. This is likely due to inhibition of MMPs

expression and activities which ultimately blocks the degradation of endothelial basement membrane proteins, a process that otherwise leads to increased permeability and poor structure and stability of the vascular endothelium. Indeed, the higher anti-MMPs activities and reduced permeability after treatment with ZOM in an *in vitro* model of DV-infection were not a function of a single factor but a combination of several factors.

Thus this study identified a new therapeutic approach which may have important role for the treatment of DV-induced permeability through modulating activities and mRNA expression gelatinolytic MMPs and TIMPs. The usual daily human ingesting of ginger of approximately 250mg to 1g has1.0-3.0% [6]-gingerol and its derivatives (O'Hara *et al*., 1998). Phytochemicals constitute a class of broadly acting and very cheap natural drugs with very low toxicity especially if given at relatively low doses. This study could serve as a primer for such investigations, which hopefully could lead to cheaper, safer and more effective anti-dengue treatment in the future.

# CHAPTER 5

# CONCLUSIONS AND RECOMMENDATIONS

## Conclusions

Based on the results presented in this study, it can be concluded that:

1. Total phenolic contents (TPC) in terms of milligram gallic acid equivalents/ gram for ZOM> ZOA > ZOC) >ZOH as determined using Folin-Ciocalteu reagent.

2. [6]-gingerol content per gram of extract for ZOM is higher compared to ZOA > ZOH as determined by high performance liquid chromatography. ZOC doesn't contain any significant amount of [6]-gingerol content.

3. [6]-gingerol has significantly lower $IC_{50}$ against Vero and MDCK cells as compared to ZOM and ZOA as shown by MTT analysis.

4. The activities of MMP-2 and to a lesser extent MMP-9 are significantly enhanced in conditioned media collected from the dengue virus infected Vero cells compared to conditioned media from non-infected cells as determined by gelatin zymography assay.

5. The mean percentage inhibition of MMP-2 and MMP-9 activities treated with ZOM is significantly higher compared to pure [6]-gingerol > ZOA and occurs in dose-dependent manner as shown by gelatin zymography assay.

6. Dengue virus infection of Vero cells results in a significant upregulation of mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2.

7. qReal time RT-PCR analyses demonstrated that mRNA expression of geltinolytic MMP-2 and MMP-9 are downregulated and the mRNA expression of TIMP-1 and TIMP-2 mRNA are upregulated in dengue virus infected Vero cells to a level significantly higher with ZOM as compared to pure [6]-gingerol > and ZOA. This suggests that *Z. officinale* rhizome extracts and pure [6]-gingerol inhibit the proteolytic activity of MMPs accompanied by an enhanced expression of their natural inhibitors, TIMPs, which may inhibit MMPs induced vascular damage.

8. Pure [6]-gingerol indeed 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.

9. *In vitro* permeability assay showed that conditioned media from dengue virus-infected Vero cells contain soluble and active factors like MMPs favoring the increased of MDCK monolayer permeability compared to CM collected from non-infected cells.

10. The permeability caused by MMPs on a monolayer of epithelial cells was significantly more reduced with ZOM as compared to pure [6]-gingerol > and ZOA in a dose dependent manner 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 that warrant further investigation.

**Recommendations**

For further studies on regulation of MMPs and TIMPs activities and expressions by *Z. officinale* rhizome extracts and pure [6]-gingerol, it is recommended that:

1. additional major phytochemicals characterized in *Z. officinale* rhizome extracts be determined, isolated and investigated for individual or even synergistic effect on DV infected cells.

2. the molecular mechanisms involved in the modulation of MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA expressions in DV infected Vero cells be determined,

particularly the effect of *Z. officinale* rhizome extracts and pure [6]-gingerol on protein expression, post-translational modification and degradation.

3. the molecular mechanisms involved in dengue virus infection induced permeability be further explored, particularly the signaling pathways involved and possible roles of ZO phytochemicals in signaling.

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# APPENDIX A

## PAPER PRESENTATION AND PUBLICATIONS

### A.1 ORAL PRESENTATION

A portion of the work has been accepted for the oral presentation during the 39th Annual Philippine Society of Biochemistry and Molecular Biology Convention at Ilocos Norte , Philippines.

Philippine Society of Biochemistry and Molecular Biology
*in cooperation with the*
Mariano Marcos State University
College of Arts and Sciences
*awards this*
*Certificate of Appreciation*
*to*
**BINITA KOIRALA SHARMA**

for being a **SPEAKER** with the topic
***"Inhibitory Effect of ZingiberofficinaleRosc. Extracts and [6]-gingerol on the Activity of MMP-2, MMP-9 Involved in Vascular Leakage In an In Vitro Model of Dengue Virus Infection"***
during the ***39th Annual PSBMB Convention*** with the theme
***"Agricultural Biotechnology & Biodiversity: Towards Sustainable Development"***
held at the Fort Ilocandia Resort Hotel, Laoag City,
Ilocos Norte, Philippines on the 29th – 30th of November, 2012.

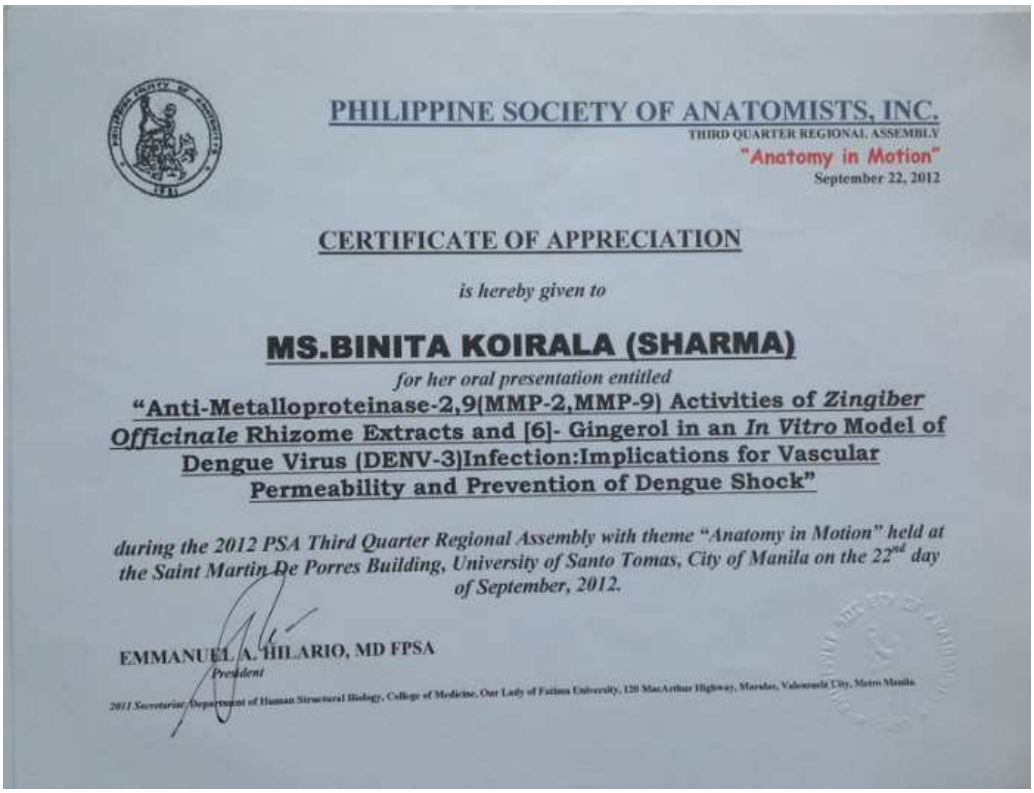
**PRIMA FE R. FRANCO, Ph.D.**
*Local Organizing Committee Chair*

**GRACIA FE B. YU, Ph.D.**
*President, PSBMB*
*Convention Chair*

**JOHN DONNIE A. RAMOS, Ph.D.**
*Convention Co-Chair*

## A.2 ORAL PRESENTATION

A portion of this work has been accepted for the oral presentation for 2012 third quarter Regional Assembly of the Philippine Society of Anatomist, Inc. Philippines.



PHILIPPINE SOCIETY OF ANATOMISTS, INC.
THIRD QUARTER REGIONAL ASSEMBLY
"Anatomy in Motion"
September 22, 2012

CERTIFICATE OF APPRECIATION

*is hereby given to*

**MS.BINITA KOIRALA (SHARMA)**

*for her oral presentation entitled*

**"Anti-Metalloproteinase-2,9(MMP-2,MMP-9) Activities of *Zingiber Officinale* Rhizome Extracts and [6]- Gingerol in an *In Vitro* Model of Dengue Virus (DENV-3)Infection:Implications for Vascular Permeability and Prevention of Dengue Shock"**

*during the 2012 PSA Third Quarter Regional Assembly with theme "Anatomy in Motion" held at the Saint Martin De Porres Building, University of Santo Tomas, City of Manila on the 22nd day of September, 2012.*

EMMANUEL A. HILARIO, MD FPSA
*President*
*2011 Secretariat: Department of Human Structural Biology, College of Medicine, Our Lady of Fatima University, 120 MacArthur Highway, Marulas, Valenzuela City, Metro Manila*

## A.3 POSTER PRESENATION

A part of this work has been included in the poster presentation in the 3rd National Convention of the Philippine Society for Cell biology, Inc.

PHILIPPINE SOCIETY FOR CELL BIOLOGY, INC.

presents this

Certificate of Appreciation

to

BINITA KOIRALA SHARMA

for serving as **PRESENTOR** for the Poster entitled ***ZINGIBER OFFICINALE*** **ROSC. EXTRACTS AND [6]-GINGEROL ON THE ACTIVITY OF MATRIC METALLOPROTEINASES-2,9 (MMP-2, MMP-9) INVOLVED IN VASCULAR LEAKAGE IN AN *INVITRO* MODEL OF DENGUE VIRUS INFECTION**
by Binita Koirala Sharma, David Klinzing, John Donnie Ramos
during the 3rd National Convention of the Philippine Society for Cell Biology, Inc.
Given this 19th day of October, 2012 at the College of Science Auditorium,
University of the Philippines, Diliman, Quezon City.

Sonia D. Jacinto, Ph.D.
PSCB President 2010-2012

Ernelea P. Cao, Ph.D.
Chair, Poster and Exhibits Committee

## A.4 PUBLICATION

A portion of the work has been published in the Asian Pacific Journal of Tropical Disease.

Asian Pac J Trop Dis 2015; 5(Suppl 1): S19-S26



HOSTED BY

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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd

Original article doi: 10.1016/S2222-1808(15)60849-0



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

Binita Koirala Sharma[1,2*], David Charles Klinzing[3,4], John Donnie Ramos[1,5]

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[2]*Department of Microbiology, Manipal College of Medical Sciences, Pokhara, Nepal*

[3]*St. Luke's Medical Center, Research and Biotechnology Division, Quezon City, Philippines*

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[5]*Research Center for the Natural and Applied Sciences, and College of Science, University of Santo Tomas, España, Manila, Philippines*

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Dengue virus
Matrix metalloproteinases
Tissue inhibitor of metalloproteinases
Vascular leakage

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.

## A.5 PUBLICATION

A portion of the work has been published in the International Journal of Pharma and Bio Science.

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

**BINITA KOIRALA SHARMA[1*] AND JOHN DONNIE A. RAMOS [1,2]**

[1]*The Graduate School, University of Santo Tomas, España, Manila, Philippines*
[2] *Research Center for the Natural and Applied Sciences, and College of Science, University of Santo Tomas, España, Manila, Philippines*

## 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 downregulate the mRNA expression of MMP-2 and MMP-9, whereas upregulate 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.

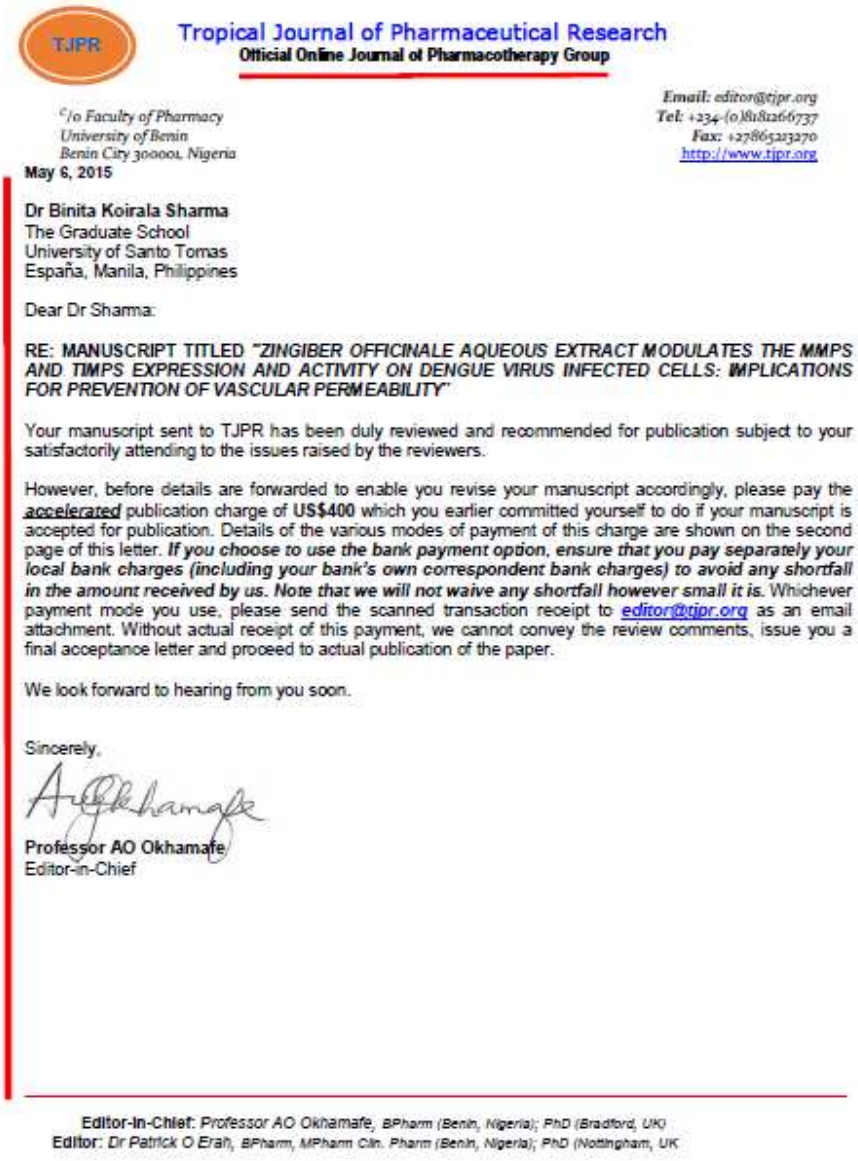
**KEYWORDS:** Dengue virus (DV), Matrix metalloproteinase (MMP), Tissue inhibitor of metalloproteinase (TIMP), [6]-gingerol

**BINITA KOIRALA SHARMA**
The Graduate School, University of Santo Tomas, España, Manila, Philippines

*Corresponding author

## A.6 PUBLICATION

A portion of the work has been accepted for publication in the Tropical Journal of Pharmaceutical Research.



TJPR

**Tropical Journal of Pharmaceutical Research**
**Official Online Journal of Pharmacotherapy Group**

*C/o Faculty of Pharmacy*
*University of Benin*
*Benin City 300001, Nigeria*

***Email:*** *editor@tjpr.org*
***Tel:*** *+234-(0)8182266737*
***Fax:*** *+27865223270*
*http://www.tjpr.org*

**May 6, 2015**

**Dr Binita Koirala Sharma**
The Graduate School
University of Santo Tomas
España, Manila, Philippines

Dear Dr Sharma:

**RE: MANUSCRIPT TITLED *"ZINGIBER OFFICINALE AQUEOUS EXTRACT MODULATES THE MMPS AND TIMPS EXPRESSION AND ACTIVITY ON DENGUE VIRUS INFECTED CELLS: IMPLICATIONS FOR PREVENTION OF VASCULAR PERMEABILITY"***

Your manuscript sent to TJPR has been duly reviewed and recommended for publication subject to your satisfactorily attending to the issues raised by the reviewers.

However, before details are forwarded to enable you revise your manuscript accordingly, please pay the ***accelerated*** publication charge of **US$400** which you earlier committed yourself to do if your manuscript is accepted for publication. Details of the various modes of payment of this charge are shown on the second page of this letter. ***If you choose to use the bank payment option, ensure that you pay separately your local bank charges (including your bank's own correspondent bank charges) to avoid any shortfall in the amount received by us. Note that we will not waive any shortfall however small it is.*** Whichever payment mode you use, please send the scanned transaction receipt to *editor@tjpr.org* as an email attachment. Without actual receipt of this payment, we cannot convey the review comments, issue you a final acceptance letter and proceed to actual publication of the paper.

We look forward to hearing from you soon.

Sincerely,

**Professor AO Okhamafe**
Editor-in-Chief

## APPENDIX B
## Certificate and Authentication of the Plant Sample

NATIONAL MUSEUM
BOTANY DIVISION
Manila

CERTIFICATION

This is to certify that the specimen/s herein listed and presented by the person/s herein noted were verified by this office.

NAME : BINITA KOIRALA (SHARMA)
SCHOOL/OFFICE/INSTITUTION : University of Santo Tomas
ADDRESS : España, Manila
PURPOSE : Dissertation

| Specimen Number | Family | Scientific Name |
|---|---|---|
| 01 | ZINGIBERACEAE | *Zingiber officinale* Rosc. |

☐ Determined by
■ Verified by

WILFREDO F. VENDIVIL, Ph. D.
Curator II
Botany Division

Date: May 07, 2012
Control No. 694
O.R. No. 1640084

## APPENDIX C

### Plant Extraction Set-up

**C.1 *Zingiber officinale* rhizomes and powder**

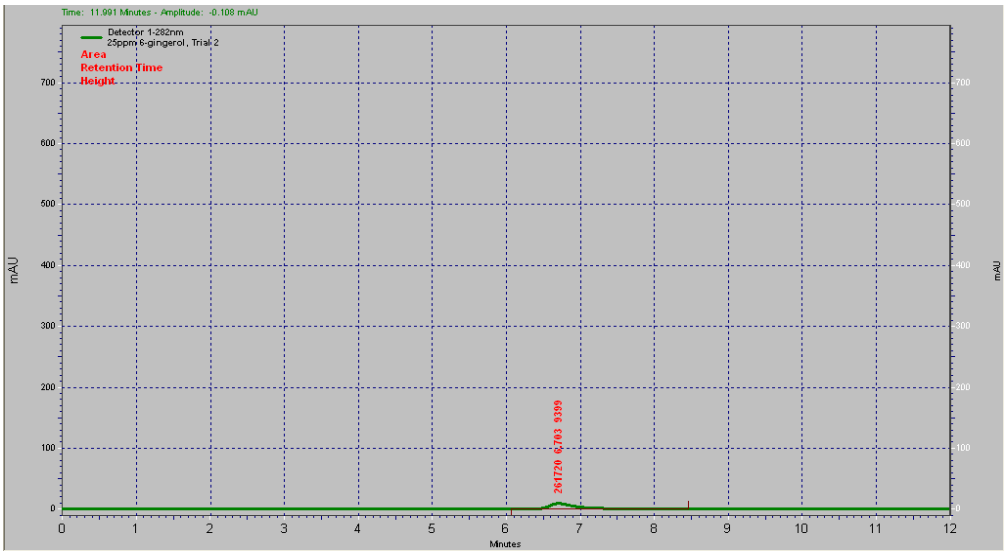

**C.2 Extracts Preparation**


**C.3 Rotary Evaporator and Lyophilizer**

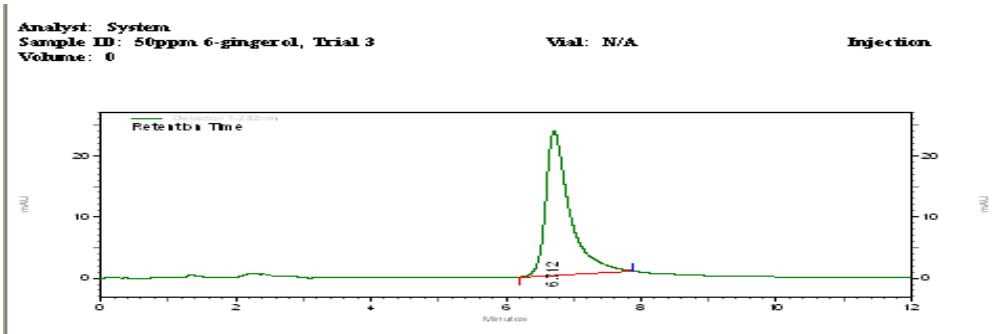

## APPENDIX D

## HPLC Analysis for [6]-gingerol Content Determination

### D.1 HPLC Chromatogram of 25.0 µg/mL [6]-gingerol standard




### D.2 HPLC Chromatogram of 50 µg/mL [6]-gingerol standard

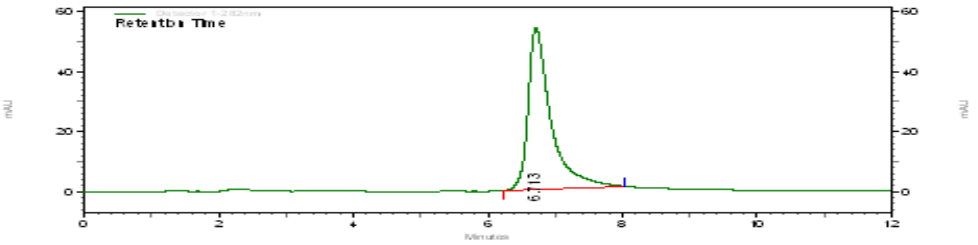



Detector 1-282nm Results (System (1/2/2003 2:19:26 AM) (Reprocessed))

| Name | Retention Time | Area | Area Percent | Integration Codes |
|---|---|---|---|---|
| | 6.712 | 577410 | 100.000 | MBM |
| Totals | | 577410 | 100.000 | |

## D.3 HPLC Chromatogram of 100 µg/mL [6]-gingerol standard

**Analyst:** System
**Sample ID:** 100ppm 6-gingerol, Trial 1 **Vial:** N/A
**Injection Volume:** 0

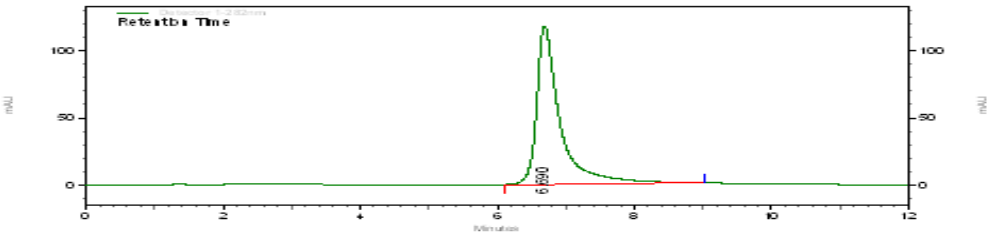



**Detector 1-282nm Results (System (1/2/2003 2:20:08 AM) (Reprocessed))**

| Name | Retention Time | Area | Area Percent | Integration Codes |
|---|---|---|---|---|
| | 6.713 | 1262776 | 100.000 | MM |
| Totals | | 1262776 | 100.000 | |

## D.4 HPLC Chromatogram of 200 µg/mL [6]-gingerol standard

**Analyst:** System
**Sample ID:** 200ppm 6-gingerol, Trial 1 **Vial:** N/A
**Injection Volume:** 0

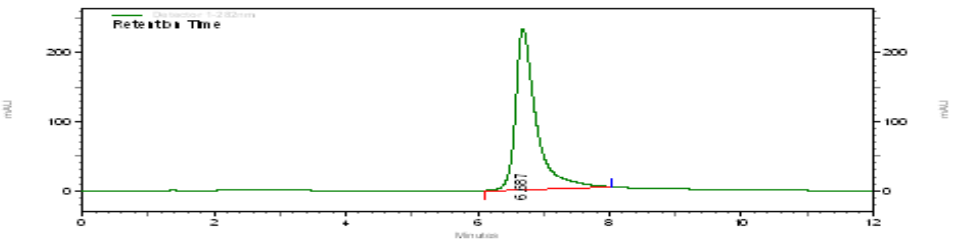



**Detector 1-282nm Results (System (1/2/2003 2:22:44 AM) (Reprocessed))**

| Name | Retention Time | Area | Area Percent | Integration Codes |
|---|---|---|---|---|
| | 6.690 | 2795555 | 100.000 | MM |
| Totals | | 2795555 | 100.000 | |

## D.5 HPLC Chromatogram of 400 µg/mL [6]-gingerol standard

**Analyst:** System
**Sample ID:** 400ppm 6-gingerol, Trial 1 **Vial:** N/A
**Injection Volume:** 0

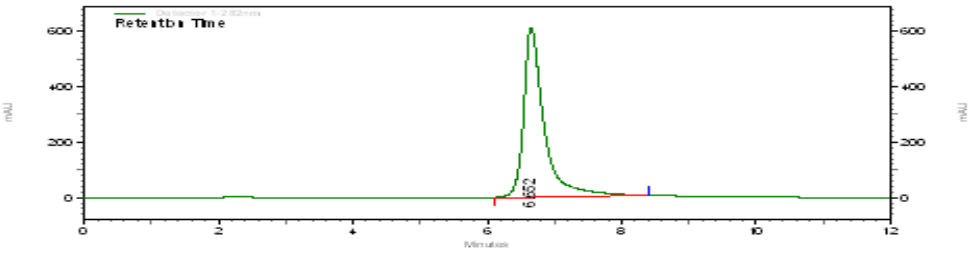



**Detector 1-282nm Results (System (1/1/2003 12:40:32 AM) (Reprocessed))**

| Name | Retention Time | Area | Area Percent | Integration Codes |
|---|---|---|---|---|
| | 6.687 | 5006225 | 100.000 | MM |
| Totals | | 5006225 | 100.000 | |

## D.6 HPLC Chromatogram of 800 µg/mL [6]-gingerol standard

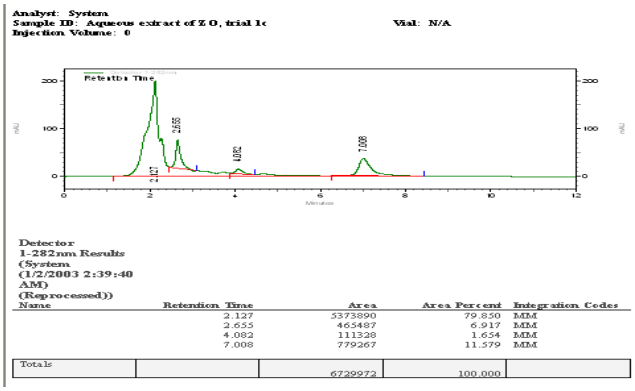
**Analyst:** System
**Sample ID:** 800ppm 6-gingerol, Trial 2 **Vial:** N/A
**Injection Volume:** 0




**Detector 1-282nm Results (System (1/2/2003 1:39:15 AM) (Reprocessed))**

| Name | Retention Time | Area | Area Percent | Integration Codes |
|---|---|---|---|---|
| | 6.652 | 12814806 | 100.000 | MM |
| Totals | | 12814806 | 100.000 | |

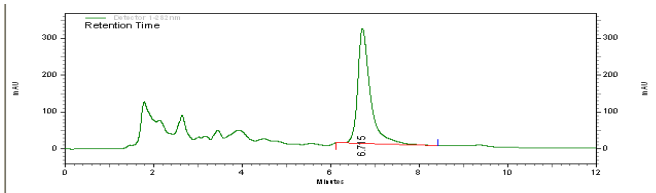
## D.7 HPLC Chromatogram of *Zingiber officinale* rhizome Aqueous Extracts (ZOA)




Detector
1-282nm Results
(System
(1/2/2003 2:39:40
AM)
(Reprocessed))

| Name | Retention Time | Area | Area Percent | Integration Codes |
|---|---|---|---|---|
| | 2.127 | 5373890 | 79.850 | MM |
| | 2.655 | 465487 | 6.917 | MM |
| | 4.082 | 111326 | 1.654 | MM |
| | 7.008 | 779267 | 11.579 | MM |
| Totals | | 6729972 | 100.000 | |

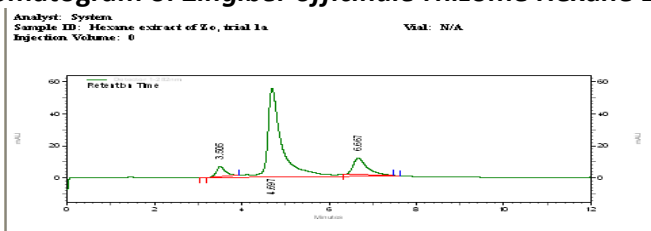
## D.8 HPLC Chromatogram of *Zingiber officinale* rhizome Methanol Extracts (ZOM)




Detector
1-282nm Results
(System
(1/1/2003
1:34:43 AM)
(Reprocessed))

| Name | Retention Time | Area | Area Percent | Integration Codes |
|---|---|---|---|---|
| | 6.715 | 6177174 | 100.000 | MM |
| Totals | | 6177174 | 100.000 | |

## D.9 HPLC Chromatogram of *Zingiber officinale* rhizome Hexane Extracts (ZOH)



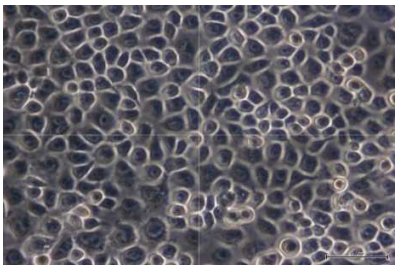

Detector
1-282nm Results
(System
(1/2/2003 3:20:47
AM)
(Reprocessed))

| Name | Retention Time | Area | Area Percent | Integration Codes |
|---|---|---|---|---|
| | 3.505 | 88689 | 5.300 | MM |
| | 4.697 | 1354447 | 80.940 | MM |
| | 6.667 | 230253 | 13.760 | mm |
| Totals | | 1673389 | 100.000 | |

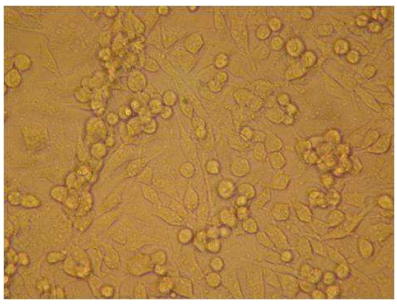
## APPENDIX E

### Cell culture

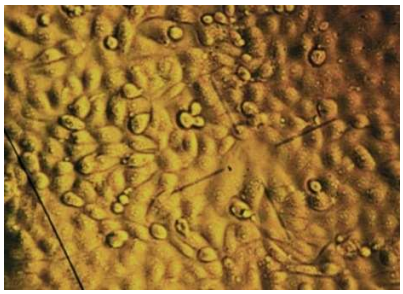
**E.1 Photograph of C6/36 cells (cloned cell line derived from larvae of *Aedes albopictus* mosquito)(X1000)**



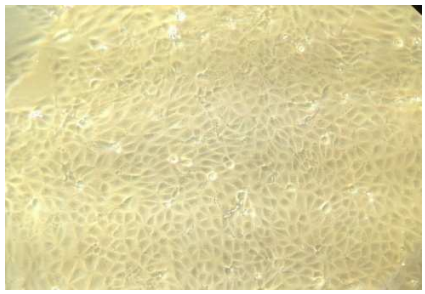
**E.2 Photograph of BHK-21 cells (baby hamster kidney derived from *Mesocricetus auratus*)(X1000)**



**E.3 Photograph of Vero cells (*Cercopithecus aethiops* kidney cells)(X10000)**

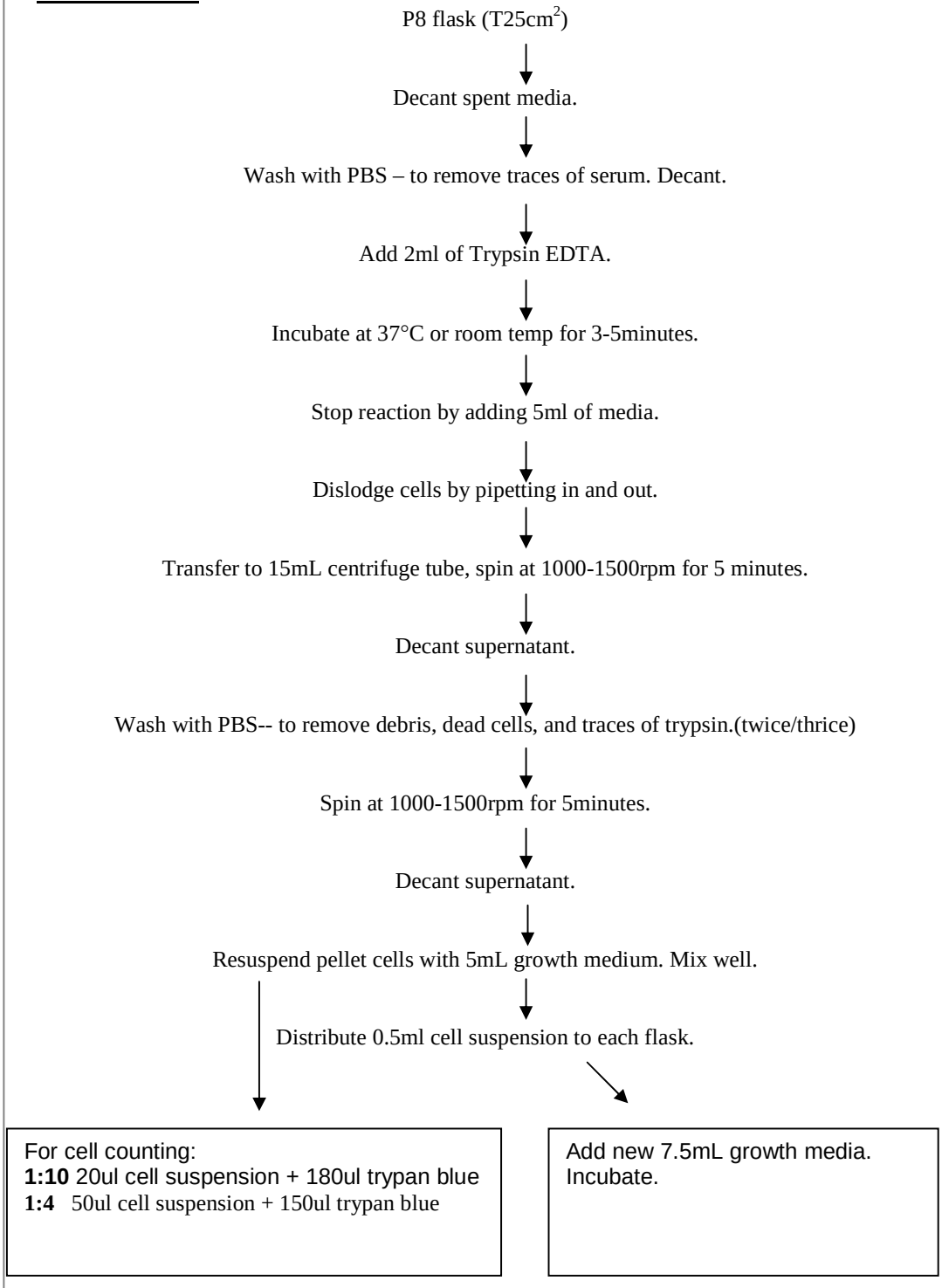


**E.4 Photograph of MDCK cells (Madin-Darbin canine Kidney cells)(X1000)**



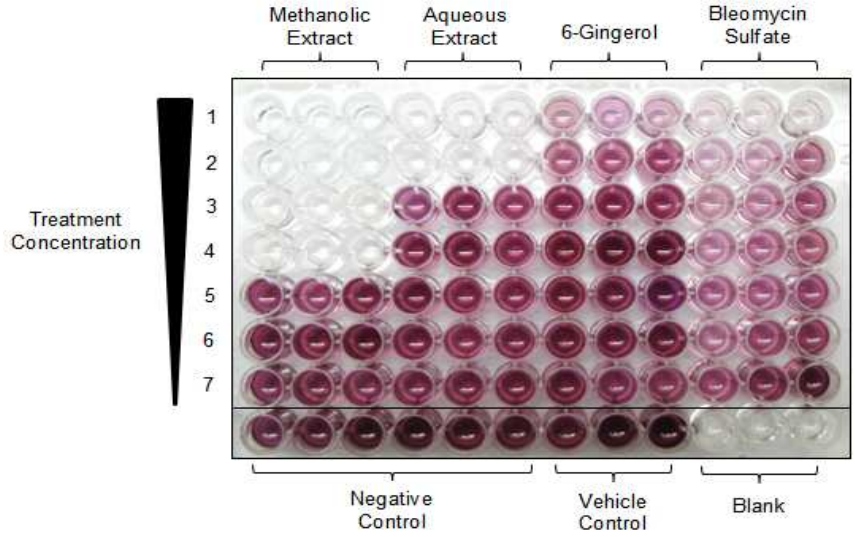
**E.5 Protocol of Research and Biotechnology Division, St. Lukes for Cell culture**

**Adherent cells**



## APPENDIX F

### F.1 Plate arrangement for MTT Cytotoxicity Assay




### F.2 Clinical Isolates of Dengue Virus in Dengue Virus Bank of RBD, St. Lukes

## APPENDIX G

### G.1 Biosafety Measures

1. Wear personal protective equipment (PPE) such as gown, gloves, mask and goggles to prevent contamination of any part of the body.
2. Wash hands properly or use alcohol-based hand rub. Dry hands with disposal paper towel.
3. Clean and disinfect working areas before and after every experiment.
4. Label waste collection containers properly and follow color codes in disposing wastes (i.e., Black: dry/recyclable; Green: wet/biodegradable; Yellow: Infectious; Orange: radioactive).
5. Read and understand the Materials and Safety Data Sheet (MSDS)* before handling chemical. Avoid contact of chemicals with skin or eyes.

* MSDS is a document that contains the chemical and physical properties, health hazard, and safe handling information for a specified chemical.

### G.2 Quality Control Measures

1. Assemble all reaction in the PCR hood using aerosol-barrier tips to avoid contamination.
2. Use dedicated reagents and filtered pipettes to prevent contamination.
3. Monitor contamination by including a negative control in each essay. The control should consist of a solution of diethylpyrocarbonate (DEPC) treated water that includes all reagents without viral DNA.
4. Confirm product using molecular weight markers.

## APPENDIX H

### DV RNA Extraction, cDNA Synthesis and RT-PCR

#### 1. Extraction of DV RNA (QIAamp®Viral RNA Mini Kit

1. Pipet 560 µl of AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.
2. Add 140 µl of serum to the Buffer AVL-carrier RNA in the microcentrifuge tube. Mix by pulse –vortexing for 15 seconds.
3. Incubate at room temperature (15-25 °C) for 10 minutes.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 56o µl of ethanol (96-100%) to the sample, and mix by the pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tube to remove drops from the lid.
6. Carefully and 630 µl solution from step 5 to the QIAamp Mini spin column without wetting the rim. Close the cap, and centrifuge at 6000xg (8000rmp) for 1 minute. Place the QIAamp spin column into a clean 2 m collection tube, and discard the tube containing the filtrate.
7. Carefully open the QIAamp Mini spin column, and repeat step 6.
8. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1.Close the cap, and centrifuge at 6000xg (8000rmp) for 1 minute. Place the QIAamp Mini spin column in a clean 2ml collection tube, and discard the tube containing the filtrate.
9. Carefully open the QIAamp Mini spin column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (2000 X g; 14,000rmp) for 3 minutes.
10. Place the QIAamp Mini spin column in a new 2 ml collection tube, and discard the old collection tube with the filtrate .Centrifuge at full speed for 1 minute.
11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube. Discard the old collection tube containing the filtrate .Carefully open the QIAamp spin column and add 60 µl of Buffer AVE equilibrated to room temperature .Close the cap, and incubate at room temperature for 1 minute .Centrifuge at 6000 X g (8000 rmp) for 1 minute.
12. Determine the nucleic acid concentration and its purity by measuring the absorbance at 260/280 nm using the NanoDrop$^{R}$ 1000 Spectrophotometer (Thermo Fisher Scientific).
13. Store viral RNA at -80 °C.

**2. cDNA Synthesis Procedure**

i. Prepare the master mix.

| Reagent | Volume per Reaction (µL) | Concentration |
|---|---|---|
| 5x Reverse Transcriptase buffer | 4.0 | 1x |
| 200 U/µL Reverse transcriptase | 0.5 | 5 U/µl |
| 20 U/µL RNAse inhibitor | 0.5 | 0.5 U/µl |
| 0.1 M DTT | 2.0 | 10 µM |
| 2.5 nM dNTP | 1.5 | 0.18 nM |
| 50 µM Random primers | 0.5 | 1.25 µM |
| Nuclease-free water | 1.0 | - |
| RNA template | 10.0 | - |
| Total | 20.0 | - |

ii. Incubate at $37^0$C for 60 minutes.

**3. RT-PCR Amplification Procedure**

| Reagent | Volume per Reaction (µL) | Concentration |
|---|---|---|
| 2x Premix Taq (Vivantis) | 12.5 (1x) | 1x |
| 25 µM Forward primer | 0.5 (500nM) | 500 nM |
| 25 µM Reverse primer | 0.5 (500 nM) | 500 nM |
| Nuclease-free water | 9.0 | - |
| cDNA template | 2.5 | - |
| Total | 25.0 | - |

**4. PCR cycles**

| | | | |
|---|---|---|---|
| Initial denaturation | 94°C | 3 minutes | |
| Denaturation | 94°C | 30 seconds | 30 cycles |
| Annealing | 56°C | 45 seconds | |
| Elongation | 72°C | 1 minute | |
| Final elongation | 72°C | 5 minutes | |

**5. Check PCR Product by running in 2% Agarose gel.**

## APPENDIX I

## Gene Expression Assay

### I.1 RNA Extraction from cells

1. Cells grown in a monolayer were harvested: Cells were lysed by trypsinization and collected as a cell pellet prior to lysis.

* To trypsinize and collect cells: The number of cells were determined. The medium was aspirated, and washed the cells with PBS. The PBS was aspirated, and 0.1–0.25% trypsin in PBS was added. After the cells detach from the dish or flask, medium (containing serum to inactivate the trypsin) was added and transfered the cells to an RNase-free centrifuge tube, and centrifuge at 300 x g for 5 min. The supernatant was completely aspirated, and proceeded to step 2.

2. Cells were disrupted by adding Buffer RLT. Vortexed to mix, and proceeded to step 3.

3. The lysate was homogenized by passing the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe and proceeded to step 4.

4. 1 volume of 70% ethanol was added to the homogenized lysate, and mixed well by pipetting.

5. 700 µl of the sample was transfered to an RNeasy spin column placed in a 2 ml collection tube. The lid was gently closed, and centrifuged for 15 s at ≥10,000 rpm. The flow-through was discarded.* The collection tube was reused in step 6.

6. 700 µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at ≥10,000 rpm to wash the spin column membrane. The flow-through was discarded.* The collection tube was reused in step 7.

7. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at ≥10,000 rpm to wash the spin column membrane. The flow-through was discarded. The collection tube was reused in step 8. Note: Buffer RPE was supplied as a concentrate so ethanol was added to Buffer RPE before use.

8. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 2 min at ≥10,000 rpm to wash the spin column membrane. This long centrifugation dried the spin column membrane, ensuring that no ethanol was carried over during RNA elution.

9. The RNeasy spin column was placed in a new 2 ml collection tube, and discarded the old collection tube with the flow-through and centrifuged at full speed for 1 min. This step was performed to eliminate any possible carryover of Buffer RPE.

10. The RNeasy spin column was placed in a new 1.5 ml collection tube. 30–50 µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at ≥10,000 rpm to elute the RNA.

## I.2 cDNA Synthesis

**I) Prepare the Master Mix**

| Reagent | Volume per Reaction (µL) | Concentration |
|---|---|---|
| Iscript transcription supermix | 4.0 | 1x |
| RNA template | Var | 1µg |
| Nuclease-free water | Var | |
| Total | 20.0 | - |

II) The reaction was incubated as follows:
Priming: 5 minutes at $25^0$C
Reverse Transcription: 30 minutes at $42^0$C
RT inactivation: 5 minutes at $85^0$C
III) The resulting cDNA was stored at $-20^0$C

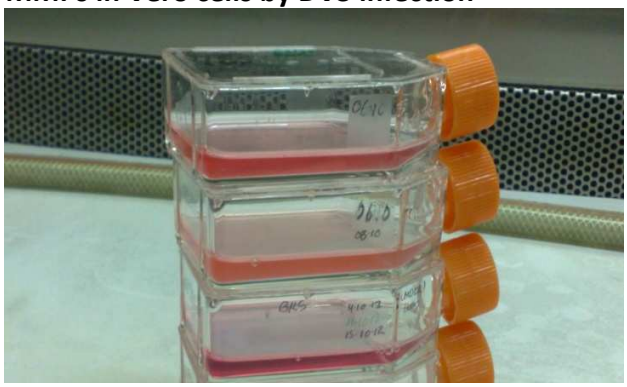
## I.3 Quantitative Real Time RT-PCR

I) Prepare the Master Mix

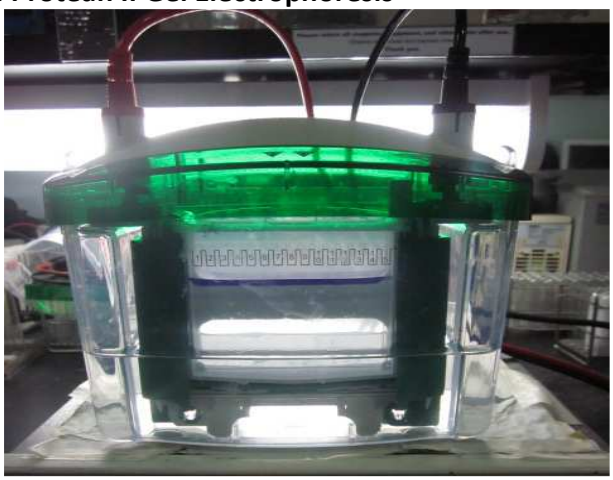
| Reagent | Volume per Reaction (µL) | Concentration |
|---|---|---|
| iQ™ Eva® Green Supermix | 10 | 1x |
| 10 µM Forward primer | 1 | 500 nM |
| 10 µM Reverse primer | 1 | 500 nM |
| Nuclease-free water | 6 | - |
| cDNA template | 2 | - |
| Total | 20.0 | - |

## APPENDIX J

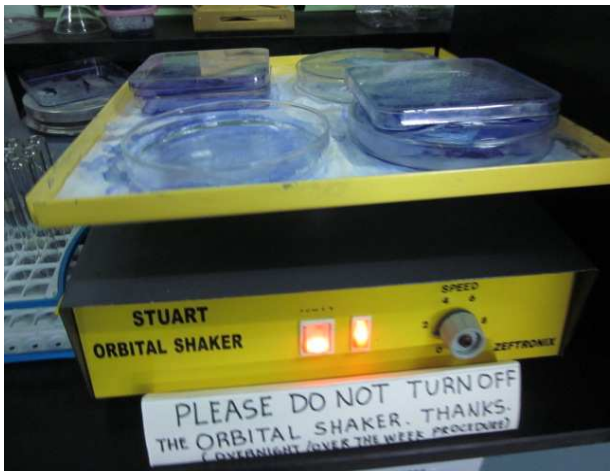
### J.1 Induction of MMPs in Vero cells by DV3 infection



### J.2 BIORAD Mini Protean II Gel Electrophoresis



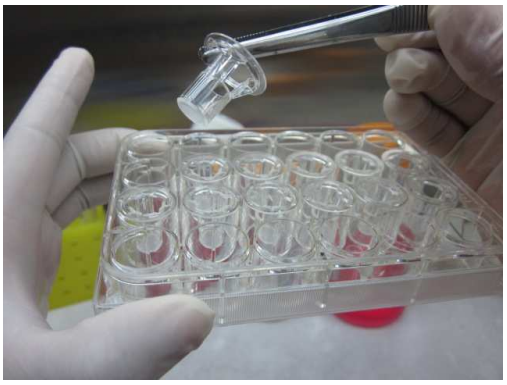
### J.3 Shaker



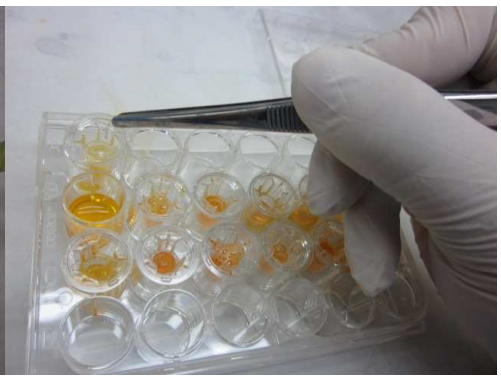
## APPENDIX K

## Permeability Assay

### K.1 Culturing MDCK cells in Transwells




### K.2 Addition of FITC-Dextran

## APPENDIX M

## STATISTICAL ANALYSIS

### M.1. Summary of retrieved dry weights of *Zingiber officinale* rhizome extracts using water, methanol, chloroform, and hexane as extraction solvents.

| Extraction Solvent | Obtained Dry Weight (g |
|---|---|
| Water | 4.790 |
| Methanol | 1.706 |
| Chloroform | 2.038 |
| Hexane | 0.410 |

Calculation of Percentage Yield:

$$\%\ Yield = \frac{obtained\ dry\ weight\ of\ extract\ in\ grams}{original\ dry\ weight\ of\ plant\ material\ used\ in\ grams}\ x\ 100$$

$$\%\ Yield\ (Water) = \frac{4.790\ g}{60.000\ g}\ x\ 100 = 7.98$$

$$\%\ Yield\ (Methanol) = \frac{1.706\ g}{60.000\ g}\ x\ 100 = 2.84$$

$$\%\ Yield\ (Chloroform) = \frac{2.038\ g}{60.000\ g}\ x\ 100 = 3.39$$

$$\%\ Yield\ (Hexane) = \frac{0.410\ g}{60.000\ g}\ x\ 100 = 0.68$$

### M.2 Statistical Analysis of Total Phenolic Contents Determination

Mean absorbance +/- SD of Three replicates (n=3)

| Extracts | Replicate | | | Average Absorbance | Phenolic Concentration (µg/mL) | mg GAE/gram of dry weight of extract |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | | | |
| Aqueous | 0.258 | 0.257 | 0.259 | 0.258 | 204.500 | 68.17±0.28 |
| Methanolic | 0.921 | 0.923 | 0.925 | 0.923 | 758.666 | 252.89 ± 0.55 |
| Chloroform | 0.219 | 0.216 | 0.215 | 0.199 | 170.055 | 56.69 ± 0.57 |
| Hexane | 0.181 | 0.182 | 0.185 | 0.181 | 141.722 | 47.24 ± 0.58 |

## M. 3 Statistical Analysis for determination of mg [6]-gingerol content per gram of extracts

### A. Statistical analyses for [6]-gingerol content using peak area

**Mean** Results +/- S.D of 3 Independent Trials in Triplicates (n=9)

| Samples | Trial | Replicate | Peak are | Avg. PA | µg/ml Conc. | Avg. Conc | s.d | C.V (%) |
|---|---|---|---|---|---|---|---|---|
| Methanolic extract | 1 | 1a | 7620605 | | | | | |
| | | 1b | 6659672 | 7022333 | 411.172 | 411.567 | 9.90523 | 2.40671 |
| | | 1c | 6786723 | | | | | |
| | 2 | 2a | 6852940 | | | | | |
| | | 2b | **6953765** | 6853592 | 401.865 | | | |
| | | 2c | 6754070 | | | | | |
| | 3 | 3a | **7354070** | | | | | |
| | | 3b | **7261756** | 7212561 | 421.664 | | | |
| | | 3c | **7021856** | | | | | |
| | | | | | | | | |
| Aqueous extract | 1 | 1a | 995838 | | | | | |
| | | 1b | 1060256 | 997865.7 | 78.8975 | | | |
| | | 1c | 937503 | | | | | |
| | 2 | 2a | 1211186 | | | | | |
| | | 2b | 1204194 | 1159003 | 87.7849 | 87.1064 | 7.8915 | 9.05961 |
| | | 2c | 1061629 | | | | | |
| | 3 | 3a | 1262213 | | | | | |
| | | 3b | 1389233 | 1283233 | 94.6367 | | | |
| | | 3c | 1198252 | | | | | |
| | | | | | | | | |
| Hexane extract | 1 | 1a | 416525 | | | | | |
| | | 1b | 404361 | 394877.3 | 45.6402 | | | |
| | | 1c | **363746** | | | | | |
| | 2 | 2a | 352162 | | | | | |
| | | 2b | 339731 | 368960 | 44.2107 | 46.3724 | 2.60612 | 5.61999 |
| | | 2c | 414987 | | | | | |
| | 3 | 3a | 469262 | | | | | |
| | | 3b | **450456** | 460623 | 49.2663 | | | |
| | | 3c | 462151 | | | | | |

| Samples | Conc (ppm) (ug/ml) | | mg 6-gingerol/gram | Avg. mg 6-gingerol/gram | s.d |
|---|---|---|---|---|---|
| Methanolic | trial1 | 411.574 | 137.1913 | | |
| extract | trial2 | 402.28 | 134.0933 | 137.32 | 1.97 |
| | trial3 | 422.0514 | 140.6838 | | |
| | | | | | |
| Aqueous | trial1 | 79.75709 | 26.5857 | | |
| extract | trial2 | 88.63224 | 29.54408 | 29.32 | 1.97 |
| | trial3 | 95.47459 | 31.82486 | | |
| | | | | | |
| Hexane | trial1 | 42.87369 | 14.29123 | | |
| extract | trial2 | 46.95403 | 15.65134 | 15.96 | 1.39 |
| | trial3 | 53.8386 | 17.9462 | | |

**B. Statistical analyses for [6]-gingerol content using retention times**

**Mean** Results +/- S.D of 3 Independent Trials in Triplicates (n=9)

| Samples | Trial | Replicate | Retention Time | Avg. RT | Avg RT | s.d | C.V (%) |
|---|---|---|---|---|---|---|---|
| Methanolic extract | 1 | 1a | 6.715 | | | | |
| | | 1b | 6.653 | 6.66833 | 6.67133 | 0.01802 | 0.27013 |
| | | 1c | 6.637 | | | | |
| | 2 | 2a | 6.68 | | | | |
| | | 2b | 6.672 | 6.69067 | | | |
| | | 2c | 6.72 | | | | |
| | 3 | 3a | 6.662 | | | | |
| | | 3b | 6.648 | 6.655 | | | |
| | | 3c | 6.655 | | | | |
| | | | | | | | |
| Aqueous extract | 1 | 1a | 6.713 | | | | |
| | | 1b | 6.597 | 6.77 | 6.73589 | 0.07613 | 1.13024 |
| | | 1c | 7 | | | | |
| | 2 | 2a | 6.513 | | | | |
| | | 2b | 6.615 | 6.64867 | | | |
| | | 2c | 6.818 | | | | |
| | 3 | 3a | 6.905 | | | | |
| | | 3b | 6.83 | 6.789 | | | |
| | | 3c | 6.632 | | | | |
| | | | | | | | |
| Hexane extract | 1 | 1a | 6.667 | 6.65233 | 6.44944 | 0.177 | 2.74438 |
| | | 1b | 6.547 | | | | |
| | | 1c | 6.743 | | | | |
| | 2 | 2a | 6.325 | 6.36933 | | | |
| | | 2b | 6.403 | | | | |
| | | 2c | 6.38 | | | | |
| | 3 | 3a | 6.298 | 6.32667 | | | |
| | | 3b | 6.325 | | | | |
| | | 3c | 6.357 | | | | |

**M. 4 Statistical Analysis of MTT Assay for Vero cells after 72 hours**

| Concentration | Aqueous Extract (ZOA)-cell viability | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Mean | S.d | SEM |
| NC | 0.92 | 0.9 | 0.91 | 0.91 | 0.01 | 0.006 |
| 25 | 0.91 | 0.86 | 0.88 | 0.88 | 0.03 | 0.015 |
| 50 | 0.85 | 0.78 | 0.84 | 0.82 | 0.04 | 0.022 |
| 100 | 0.75 | 0.79 | 0.73 | 0.76 | 0.03 | 0.018 |
| 200 | 0.64 | 0.58 | 0.61 | 0.61 | 0.03 | 0.017 |
| 400 | 0.43 | 0.384 | 0.48 | 0.43 | 0.05 | 0.028 |
| 800 | 0.247 | 0.21 | 0.283 | 0.25 | 0.04 | 0.021 |
| 1600 | 0.103 | 0.143 | 0.125 | 0.12 | 0.02 | 0.012 |
| PC | 0.005 | 0.009 | 0.011 | 0.01 | 0.00 | 0.002 |

| Concentration | [6]-gingerol-cell viability | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Mean | S.d | SEM |
| NC | 0.82 | 0.853 | 0.894 | 0.86 | 0.04 | 0.021 |
| 25 | 0.793 | 0.714 | 0.786 | 0.76 | 0.04 | 0.025 |
| 50 | 0.742 | 0.683 | 0.693 | 0.71 | 0.03 | 0.018 |
| 100 | 0.601 | 0.535 | 0.582 | 0.57 | 0.03 | 0.020 |
| 200 | 0.403 | 0.483 | 0.482 | 0.46 | 0.05 | 0.027 |
| 400 | 0.231 | 0.284 | 0.256 | 0.26 | 0.03 | 0.015 |
| 800 | 0.147 | 0.198 | 0.183 | 0.18 | 0.03 | 0.015 |
| 1600 | 0.103 | 0.097 | 0.052 | 0.08 | 0.03 | 0.016 |
| PC | 0.005 | 0.009 | 0.011 | 0.01 | 0.00 | 0.002 |

| Concentration | Methanol Extract (ZOM)-cell viability | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Mean | S.d | SEM |
| NC | 0.891 | 0.86 | 0.93 | 0.89 | 0.04 | 0.020 |
| 25 | 0.834 | 0.873 | 0.792 | 0.83 | 0.04 | 0.023 |
| 50 | 0.734 | 0.768 | 0.794 | 0.77 | 0.03 | 0.017 |
| 100 | 0.615 | 0.699 | 0.643 | 0.65 | 0.04 | 0.025 |
| 200 | 0.518 | 0.469 | 0.428 | 0.47 | 0.05 | 0.026 |
| 400 | 0.256 | 0.317 | 0.389 | 0.32 | 0.07 | 0.038 |
| 800 | 0.117 | 0.125 | 0.178 | 0.14 | 0.03 | 0.019 |
| 1600 | 0.056 | 0.062 | 0.068 | 0.06 | 0.01 | 0.003 |
| PC | 0.003 | 0.002 | 0.01 | 0.01 | 0.00 | 0.003 |

**M. 5 Statistical Analysis of MTT Assay for MDCK cells after 72 hours**

| Concn. | Aqueous Extract (ZOA)-cell viability | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Mean | S.d | SEM |
| NC | 0.913 | 0.932 | 0.951 | 0.93 | 0.02 | 0.011 |
| 25 | 0.892 | 0.832 | 0.885 | 0.87 | 0.03 | 0.019 |
| 50 | 0.862 | 0.802 | 0.832 | 0.83 | 0.03 | 0.017 |
| 100 | 0.712 | 0.792 | 0.763 | 0.76 | 0.04 | 0.023 |
| 200 | 0.613 | 0.582 | 0.552 | 0.58 | 0.03 | 0.018 |
| 400 | 0.394 | 0.453 | 0.412 | 0.42 | 0.03 | 0.017 |
| 800 | 0.215 | 0.26 | 0.31 | 0.26 | 0.05 | 0.027 |
| 1600 | 0.108 | 0.182 | 0.179 | 0.16 | 0.04 | 0.024 |
| 3200 | 0.027 | 0.032 | 0.018 | 0.03 | 0.01 | 0.004 |

| Concn. | [6]-gingerol-cell viability | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Mean | S.d | SEM |
| 12.5 | 0.838 | 0.856 | 0.782 | 0.83 | 0.04 | 0.022 |
| 25 | 0.792 | 0.826 | 0.754 | 0.79 | 0.04 | 0.021 |
| 50 | 0.68 | 0.719 | 0.723 | 0.71 | 0.02 | 0.014 |
| 100 | 0.506 | 0.541 | 0.569 | 0.54 | 0.03 | 0.018 |
| 200 | 0.314 | 0.319 | 0.375 | 0.34 | 0.03 | 0.020 |
| 400 | 0.125 | 0.198 | 0.126 | 0.15 | 0.04 | 0.024 |
| 800 | 0.02 | 0.015 | 0.09 | 0.04 | 0.04 | 0.024 |
| 1600 | 0.01 | 0.012 | 0.01 | 0.01 | 0.00 | 0.001 |
| 3200 | 0.007 | 0.008 | 0.015 | 0.01 | 0.00 | 0.003 |

| Concn. | Methanol Extract (ZOM)-cell viability | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Mean | S.d | SEM |
| 12.5 | 0.895 | 0.916 | 0.957 | 0.92 | 0.03 | 0.018 |
| 25 | 0.817 | 0.8934 | 0.825 | 0.85 | 0.04 | 0.024 |
| 50 | 0.749 | 0.793 | 0.818 | 0.79 | 0.03 | 0.020 |
| 100 | 0.593 | 0.735 | 0.617 | 0.65 | 0.08 | 0.044 |
| 200 | 0.494 | 0.386 | 0.414 | 0.43 | 0.06 | 0.032 |
| 400 | 0.217 | 0.265 | 0.316 | 0.27 | 0.05 | 0.029 |
| 800 | 0.184 | 0.149 | 0.093 | 0.14 | 0.05 | 0.027 |
| 1600 | 0.084 | 0.03 | 0.021 | 0.05 | 0.03 | 0.020 |
| 3200 | 0.003 | 0.009 | 0.018 | 0.01 | 0.01 | 0.004 |

**M.6 Statistical Analysis of Gelatin Zymograpy for non-infected, DV-infected and EDTA treated –Conditioned media collectd from vero cells**

| Relative MMP-2 activity (%) | | | | Avg | s.d | SEM |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | | | |
| non-infected -CM | 5.204 | 4.544 | 3.122 | 4.290 | 1.064 | 0.614 |
| DV-infected-CM | 96.006 | 100.000 | 80.740 | 92.248 | 10.165 | 5.869 |
| DV-CM-EDTA | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| Relative MMP-9 activity (%) | | | | | | |
| | Trial 1 | Trial 2 | Trial 3 | Avg | s.d | SEM |
| non-infected -CM | 0.627 | 0.877 | 1.862 | 1.122 | 0.653 | 0.377 |
| DV-infected-CM | 28.166 | 32.560 | 22.614 | 27.780 | 4.984 | 2.877 |
| DV-CM-EDTA | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

**Multi-factor Analysis of Variance**

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | Table Analyzed | -VE, +VE, edta | | | | |
| 2 | | | | | | |
| 3 | Two-way RM ANOVA | Matching: Both factors | | | | |
| 4 | Alpha | 0.05 | | | | |
| 5 | | | | | | |
| 6 | Source of Variation | % of total variation | P value | P value summary | Significant? | |
| 7 | Tests | 67.93 | 0.0001 | *** | Yes | |
| 8 | MMPs | 11.27 | 0.0037 | ** | Yes | |
| 9 | Interaction: Tests x MMPs | 19.52 | < 0.0001 | **** | Yes | |
| 10 | Interaction: Tests x Subjects | 0.7286 | | | | |
| 11 | Interaction: MMPs x Subjects | 0.08457 | | | | |
| 12 | Subjects | 0.3864 | | | | |
| 13 | | | | | | |
| 14 | ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| 15 | Tests | 13787 | 2 | 6893 | F (2, 4) = 186.5 | P = 0.0001 |
| 16 | MMPs | 2287 | 1 | 2287 | F (1, 2) = 266.5 | P = 0.0037 |
| 17 | Interaction: Tests x MMPs | 3962 | 2 | 1981 | F (2, 4) = 495.5 | P < 0.0001 |
| 18 | Interaction: Tests x Subjects | 147.9 | 4 | 36.97 | | |
| 19 | Interaction: MMPs x Subjects | 17.16 | 2 | 8.582 | | |
| 20 | Subjects | 78.43 | 2 | 39.21 | | |
| 21 | Residual | 15.99 | 4 | 3.998 | | |

| Number of families | 2 | | | | |
|---|---|---|---|---|---|
| Number of comparisons per family | 3 | | | | |
| Alpha | 0.05 | | | | |
| Tukey's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
| **MMP-2** | | | | | |
| DV-VE vs. DV+VE | -87.96 | -93.78 to -82.14 | Yes | **** | < 0.0001 |
| DV-VE vs. EDTA | 4.290 | -1.528 to 10.11 | No | ns | 0.1197 |
| DV+VE vs. EDTA | 92.25 | 86.43 to 98.07 | Yes | **** | < 0.0001 |
| **MMP-9** | | | | | |
| DV-VE vs. DV+VE | -26.66 | -32.48 to -20.84 | Yes | *** | 0.0002 |
| DV-VE vs. EDTA | 1.122 | -4.696 to 6.940 | No | ns | 0.7831 |
| DV+VE vs. EDTA | 27.78 | 21.96 to 33.60 | Yes | *** | 0.0002 |

**M.7 Statistical Analysis of Gelatin Zymograpy for relative MMP-2 activity in conditioned media treated with extracts ( ZOA, ZOM) and [6]-gingerol**

**A. Aqueous extract (ZOA) vs MMP-2 activity**

| | RELATIVE MMP-2 ACTIVITY (%) | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | AVG | SD | SEM |
| DV | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| DV+ ZOA 6.25 µg/ml | 86.00 | 93.45 | 92.15 | 90.53 | 3.98 | 2.30 |
| DV+ ZOA 12.5 µg/ml | 87.34 | 82.57 | 91.28 | 87.06 | 4.36 | 2.52 |
| DV+ ZOA 25 µg/ml | 18.00 | 25.00 | 20.00 | 21.00 | 3.61 | 2.08 |
| DV+ ZOA 50 µg/ml | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| DV+ EGCG 100µM | 30.65 | 40.16 | 32.10 | 34.30 | 5.12 | 2.96 |

**Multi-Factor analysis of variance**

| ANOVA summary | |
|---|---|
| F | 226 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.99 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 19905 | 5 | 3981 | F (5, 12) = 226 | P < 0.0001 |
| Residual (within columns) | 211 | 12 | 18 | | |
| Total | 20116 | 17 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | Number of families | 1 | | | | |
| 2 | Number of comparisons per family | 5 | | | | |
| 3 | Alpha | 0.05 | | | | |
| 4 | | | | | | |
| 5 | Dunnett's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
| 6 | | | | | | |
| 7 | DV+ ZOA (6.25µg/ml) vs. DV | -5.3 | -15 to 4.6 | No | ns | 0.4260 |
| 8 | DV+ ZOA (12.5µg/ml) vs. DV | -11 | -21 to -1.4 | Yes | * | 0.0243 |
| 9 | DV+ ZOA (25µg/ml) vs. DV | -62 | -72 to -52 | Yes | **** | < 0.0001 |
| 10 | DV+ ZOA (50µg/ml) vs. DV | -84 | -94 to -74 | Yes | **** | < 0.0001 |
| 11 | DV+EGCG(100µM) vs. DV | -66 | -76 to -56 | Yes | **** | < 0.0001 |
| 12 | | | | | | |

**B. Methanol extract (ZOM) vs MMP-2 activity**

| | RELATIVE MMP-2 ACTIVITY (%) | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | AVG | SD | SEM |
| DV | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| DV+ ZOM 6.25 µg/ml | 82 | 82.00 | 86.00 | 74.00 | 80.67 | 6.11 |
| DV+ ZOM 12.5 µg/ml | 56 | 56.00 | 59.00 | 63.00 | 59.33 | 3.51 |
| DV+ ZOM 25 µg/ml | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| DV+ ZOM 50 µg/ml | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| DV+ EGCG 100µM | 30.65 | 30.65 | 40.16 | 32.10 | 34.30 | 5.12 |

**Multi-Factor analysis of variance**

| Table Analyzed | | | | | |
|---|---|---|---|---|---|
| ANOVA summary | | | | | |
| F | | | | 411 | |
| P value | | | | < 0.0001 | |
| P value summary | | | | **** | |
| Are differences among means statistically significant? (P < 0.05) | | | | Yes | |
| R square | | | | 0.99 | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Treatment (between columns) | 25992 | 5 | 5198 | F (5, 12) = 411 | P < 0.0001 |
| Residual (within columns) | 152 | 12 | 13 | | |
| Total | 26143 | 17 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | Number of families | 1 | | | | |
| 2 | Number of comparisons per family | 5 | | | | |
| 3 | Alpha | 0.05 | | | | |
| 4 | | | | | | |
| 5 | Dunnett's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
| 6 | | | | | | |
| 7 | DV+ ZOM (6.25µg/ml) vs. DV | -19 | -28 to -11 | Yes | *** | 0.0001 |
| 8 | DV+ ZOM (12.5µg/ml) vs. DV | -41 | -49 to -32 | Yes | **** | < 0.0001 |
| 9 | DV+ ZOM (25µg/ml) vs. DV | -100 | -108 to -92 | Yes | **** | < 0.0001 |
| 10 | DV+ ZOM (50µg/ml) vs. DV | -100 | -108 to -92 | Yes | **** | < 0.0001 |
| 11 | DV+EGCG(100µM) vs. D | -66 | -74 to -57 | Yes | **** | < 0.0001 |

C. **[6]-gingerol vs MMP-2 activity**

| | RELATIVE MMP-2 ACTIVITY (%) | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | AVG | SD | SEM |
| DV | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| DV+ [6]-gingerol 6.25 µg/ml | 86.00 | 93.45 | 92.15 | 90.53 | 3.98 | 2.30 |
| DV+ [6]-gingerol 6.25 µg/ml | 87.34 | 82.57 | 91.28 | 87.06 | 4.36 | 2.52 |
| DV+ [6]-gingerol 6.25 µg/ml | 18.00 | 25.00 | 20.00 | 21.00 | 3.61 | 2.08 |
| DV+ [6]-gingerol 6.25 µg/ml | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| DV+ EGCG 100µM | 30.65 | 40.16 | 32.10 | 34.30 | 5.12 | 2.96 |

**Multi-Factor analysis of variance**

| Table Analyzed | | | | | |
|---|---|---|---|---|---|
| | | | | | |
| ANOVA summary | | | | | |
| F | | | | 510 | |
| P value | | | | < 0.0001 | |
| P value summary | | | | **** | |
| Are differences among means statistically significant? (P < 0.05) | | | | Yes | |
| R square | | | | 1 | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Treatment (between columns) | 27121 | 5 | 5424 | F (5, 12) = 510 | P < 0.0001 |
| Residual (within columns) | 128 | 12 | 11 | | |
| Total | 27249 | 17 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | Number of families | 1 | | | | |
| 2 | Number of comparisons per family | 5 | | | | |
| 3 | Alpha | 0.05 | | | | |
| 4 | | | | | | |
| 5 | Dunnett's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
| 6 | | | | | | |
| 7 | DV+ [6]-gingerol (6.25µg/ml) vs. DV | -9.5 | -17 to -1.7 | Yes | * | 0.0157 |
| 8 | DV+ [6]-gingerol (12.5µg/ml) vs. DV | -13 | -21 to -5.2 | Yes | ** | 0.0016 |
| 9 | DV+ [6]-gingerol (25µg/ml) vs. DV | -79 | -87 to -71 | Yes | **** | < 0.0001 |
| 10 | DV+ [6]-gingerol (50µg/ml) vs. DV | -100 | -108 to -92 | Yes | **** | < 0.0001 |
| 11 | DV+EGCG(100µM) vs. D | -68 | -76 to -61 | Yes | **** | < 0.0001 |
| 12 | | | | | | |

**M.8 Statistical Analysis of Gelatin Zymograpy for relative MMP-9 activity in conditioned media treated with extracts (ZOA, ZOM) and [6]-gingerol**

A. **ZOA vs MMP-9 activity**

| | RELATIVE MMP-9 ACTIVITY (%) | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | AVG | SD | SEM |
| DV | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| DV+ ZOA 6.25 µg/ml | 97.00 | 96.00 | 93.00 | 95.33 | 2.08 | 1.20 |
| DV+ ZOA 12.5 µg/ml | 92.00 | 99.00 | 94.00 | 95.00 | 3.61 | 2.08 |
| DV+ ZOA 25 µg/ml | 54.00 | 63.00 | 58.00 | 58.33 | 4.51 | 2.60 |
| DV+ ZOA 50 µg/ml | 34.00 | 39.00 | 29.00 | 34.00 | 5.00 | 2.89 |
| DV+ EGCG 100µM | 28.35 | 36.00 | 21.00 | 28.45 | 7.50 | 4.33 |

**Multi-Factor analysis of variance**

| Table Analyzed | | | | | |
|---|---|---|---|---|---|
| | | | | | |
| ANOVA summary | | | | | |
| F | | | | 161 | |
| P value | | | | < 0.0001 | |
| P value summary | | | | **** | |
| Are differences among means statistically significant? (P < 0.05) | | | | Yes | |
| R square | | | | 0.99 | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Treatment (between columns) | 15936 | 5 | 3187 | F (5, 12) = 161 | P < 0.0001 |
| Residual (within columns) | 238 | 12 | 20 | | |
| Total | 16174 | 17 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | Number of families | 1 | | | | |
| 2 | Number of comparisons per family | 5 | | | | |
| 3 | Alpha | 0.05 | | | | |
| 4 | | | | | | |
| 5 | Dunnett's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
| 6 | | | | | | |
| 7 | DV+6.25 µg/ml ZOA vs. DV-infected | -4.7 | -15 to 5.9 | No | ns | 0.5921 |
| 8 | DV+12.5 µg/ml ZOA vs. DV-infected | -5.0 | -16 to 5.5 | No | ns | 0.5337 |
| 9 | DV+25 µg/ml ZOA vs. DV-infected | -42 | -52 to -31 | Yes | **** | < 0.0001 |
| 10 | DV+50 µg/mlZOA vs. DV-infected | -66 | -77 to -55 | Yes | **** | < 0.0001 |
| 11 | DV+100 µM EGCG vs. DV-infected | -72 | -82 to -61 | Yes | **** | < 0.0001 |

B. **ZOM vs MMP-9 activity**

| | RELATIVE MMP-9 ACTIVITY (%) | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | AVG | SD | SEM |
| DV | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| DV+ ZOM 6.25 µg/ml | 81 | 81.00 | 89.00 | 84.00 | 84.67 | 4.04 |
| DV+ ZOM 12.5 µg/ml | 61 | 61.00 | 65.00 | 69.00 | 65.00 | 4.00 |
| DV+ ZOM 25 µg/ml | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| DV+ ZOM 50 µg/ml | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| DV+ EGCG 100µM | 28.35 | 36.00 | 21.00 | 28.45 | 7.50 | 4.33 |

**Multi-Factor analysis of variance**

| Table Analyzed | | | | | |
|---|---|---|---|---|---|
| | | | | | |
| ANOVA summary | | | | | |
| F | | | | | 161 |
| P value | | | | | < 0.0001 |
| P value summary | | | | | **** |
| Are differences among means statistically significant? (P < 0.05) | | | | | Yes |
| R square | | | | | 0.99 |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Treatment (between columns) | 15936 | 5 | 3187 | F (5, 12) = 161 | P < 0.0001 |
| Residual (within columns) | 238 | 12 | 20 | | |
| Total | 16174 | 17 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | Number of families | 1 | | | | |
| 2 | Number of comparisons per family | 5 | | | | |
| 3 | Alpha | 0.05 | | | | |
| 4 | | | | | | |
| 5 | Dunnett's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
| 6 | | | | | | |
| 7 | DV-infected vs. DV+6.25 µg/ml ZOM | 15.33 | 6.231 to 24.44 | Yes | ** | 0.0016 |
| 8 | DV-infected vs. DV+12.5 µg/ml ZOM | 35.00 | 25.90 to 44.10 | Yes | **** | < 0.0001 |
| 9 | DV-infected vs. DV+25 µg/ml ZOM | 100.0 | 90.90 to 109.1 | Yes | **** | < 0.0001 |
| 10 | DV-infected vs. DV+50 µg/ml ZOM | 100.0 | 90.90 to 109.1 | Yes | **** | < 0.0001 |
| 11 | DV-infected vs. DV+100 µM EGCG | 71.55 | 62.45 to 80.65 | Yes | **** | < 0.0001 |

C. **[6]-gingerol vs MMP-9 activity**

| | RELATIVE MMP-9 ACTIVITY (%) | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | AVG | SD | SEM |
| DV | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| DV+ [6]-gingerol 6.25 µg/ml | 97.00 | 98.00 | 92.00 | 95.67 | 3.21 | 1.86 |
| DV+ [6]-gingerol 6.25 µg/ml | 87.00 | 89.00 | 91.00 | 89.00 | 2.00 | 1.15 |
| DV+ [6]-gingerol 6.25 µg/ml | 38.00 | 47.00 | 42.00 | 42.33 | 4.51 | 2.60 |
| DV+ [6]-gingerol 6.25 µg/ml | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| DV+ EGCG 100µM | 28.35 | 36.00 | 21.00 | 28.45 | 7.50 | 4.33 |

**Multi-Factor analysis of variance**

| Table Analyzed | |
|---|---|
| | |
| ANOVA summary | |
| F | 378 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.99 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 25852 | 5 | 5170 | F (5, 12) = 341 | P < 0.0001 |
| Residual (within columns) | 182 | 12 | 15 | | |
| Total | 26033 | 17 | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | Number of families | 1 | | | | | |
| 2 | Number of comparisons per family | 5 | | | | | |
| 3 | Alpha | 0.05 | | | | | |
| 4 | | | | | | | |
| 5 | Dunnett's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value | ?-A |
| 6 | | | | | | | |
| 7 | DV+6.25 µg/ml [6]-gingerol vs. DV-infected | -4.3 | -12 to 3.2 | No | ns | 0.3683 | B |
| 8 | DV+12.5 µg/ml [6]-gingerol vs. DV-infected | -11 | -19 to -3.5 | Yes | ** | 0.0048 | C |
| 9 | DV+25 µg/ml [6]-gingerol vs. DV-infected | -58 | -65 to -50 | Yes | **** | < 0.0001 | D |
| 10 | DV+50 µg/ml [6]-gingerol vs. DV-infected | -100 | -108 to -92 | Yes | **** | < 0.0001 | E |
| 11 | DV+100 µM EGCG vs. DV-infected | -66 | -73 to -58 | Yes | **** | < 0.0001 | F |

**M.9 Statistical Analysis of gene expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 for non-infected and DV-infected Vero cells**

| Relative Fold of Induction | | | | | | |
|---|---|---|---|---|---|---|
| Genes | | Non infected | | DV-infected | | |
| MMP-2 | 1.00 | 1.00 | 1.00 | 9.58 | 10.27 | 8.46 |
| MMP-9 | 1.00 | 1.10 | 1.20 | 5.66 | 6.32 | 5.58 |
| TIMP-1 | 1.00 | 0.80 | 0.90 | 2.04 | 2.17 | 1.97 |
| TIMP-2 | 1.00 | 1.00 | 1.00 | 3.25 | 3.18 | 2.58 |

| Table Analyzed | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | | | | | |
| Alpha | 0.05 | | | | |
| Source of Variation | % of total variation | P value | P value summary | Significant? | |
| Interaction | 24.00 | < 0.0001 | **** | Yes | |
| Genes | 25.13 | < 0.0001 | **** | Yes | |
| Experimental group | 49.72 | < 0.0001 | **** | Yes | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 48.41 | 3 | 16.14 | F (3, 16) = 110.8 | P < 0.0001 |
| Genes | 50.68 | 3 | 16.89 | F (3, 16) = 116.0 | P < 0.0001 |
| Experimental group | 100.3 | 1 | 100.3 | F (1, 16) = 688.5 | P < 0.0001 |
| Residual | 2.331 | 16 | 0.1457 | | |
| Number of missing values | 0 | | | | |

| Number of families | 1 | | | |
|---|---|---|---|---|
| Number of comparisons per family | 28 | | | |
| Alpha | 0.05 | | | |
| Tukey's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| MMP-2:Non infected vs. MMP-2:DV-infected | -8.437 | Yes | **** | < 0.0001 |
| MMP-2:Non infected vs. MMP-9:Non infected | -0.1000 | No | ns | > 0.9999 |
| MMP-2:Non infected vs. MMP-9:DV-infected | -4.853 | Yes | **** | < 0.0001 |
| MMP-2:Non infected vs. TIMP-1:Non infected | 0.1000 | No | ns | > 0.9999 |
| MMP-2:Non infected vs. TIMP-1:DV-infected | -1.060 | No | ns | 0.0560 |
| MMP-2:Non infected vs. TIMP-2:Non infected | 0.0 | No | ns | > 0.9999 |
| MMP-2:Non infected vs. TIMP-2:DV-infected | -2.003 | Yes | *** | 0.0002 |
| MMP-2:DV-infected vs. MMP-9:Non infected | 8.337 | Yes | **** | < 0.0001 |
| MMP-2:DV-infected vs. MMP-9:DV-infected | 3.583 | Yes | **** | < 0.0001 |
| MMP-2:DV-infected vs. TIMP-1:Non infected | 8.537 | Yes | **** | < 0.0001 |
| MMP-2:DV-infected vs. TIMP-1:DV-infected | 7.377 | Yes | **** | < 0.0001 |
| MMP-2:DV-infected vs. TIMP-2:Non infected | 8.437 | Yes | **** | < 0.0001 |
| MMP-2:DV-infected vs. TIMP-2:DV-infected | 6.433 | Yes | **** | < 0.0001 |
| MMP-9:Non infected vs. MMP-9:DV-infected | -4.753 | Yes | **** | < 0.0001 |
| MMP-9:Non infected vs. TIMP-1:Non infected | 0.2000 | No | ns | 0.9975 |
| MMP-9:Non infected vs. TIMP-1:DV-infected | -0.9600 | No | ns | 0.1004 |
| MMP-9:Non infected vs. TIMP-2:Non infected | 0.1000 | No | ns | > 0.9999 |
| MMP-9:Non infected vs. TIMP-2:DV-infected | -1.903 | Yes | *** | 0.0003 |
| MMP-9:DV-infected vs. TIMP-1:Non infected | 4.953 | Yes | **** | < 0.0001 |
| MMP-9:DV-infected vs. TIMP-1:DV-infected | 3.793 | Yes | **** | < 0.0001 |
| MMP-9:DV-infected vs. TIMP-2:Non infected | 4.853 | Yes | **** | < 0.0001 |
| MMP-9:DV-infected vs. TIMP-2:DV-infected | 2.850 | Yes | **** | < 0.0001 |
| TIMP-1:Non infected vs. TIMP-1:DV-infected | -1.160 | Yes | * | 0.0305 |
| TIMP-1:Non infected vs. TIMP-2:Non infected | -0.1000 | No | ns | > 0.9999 |
| TIMP-1:Non infected vs. TIMP-2:DV-infected | -2.103 | Yes | *** | 0.0001 |
| TIMP-1:DV-infected vs. TIMP-2:Non infected | 1.060 | No | ns | 0.0560 |
| TIMP-1:DV-infected vs. TIMP-2:DV-infected | -0.9433 | No | ns | 0.1103 |
| TIMP-2:Non infected vs. TIMP-2:DV-infected | -2.003 | Yes | *** | 0.0002 |

## M.10 Statistical Analysis of gene expression of MMP-2 for non-infected, DV-infected and DV-infected-Treated Vero cells

### A. ZOA vs MMP-2 expression

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.15 | 1.00 | 0.91 | 0.88 | 0.56 | 0.15 | 0.87 |
| 2 | 0.08 | 1.00 | 0.97 | 0.83 | 0.59 | 0.19 | 0.75 |
| 3 | 0.09 | 1.00 | 0.96 | 0.89 | 0.54 | 0.23 | 0.84 |
| Mean | 0.11 | 1.00 | 0.95 | 0.87 | 0.56 | 0.19 | 0.82 |
| S.D | 0.04 | 0.00 | 0.03 | 0.03 | 0.03 | 0.04 | 0.06 |
| SEM | 0.02 | 0.00 | 0.02 | 0.02 | 0.02 | 0.02 | 0.04 |

| ANOVA summary | |
|---|---|
| F | 291 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.99 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 2.4 | 6 | 0.40 | F (6, 14) = 291 | P < 0.0001 |
| Residual (within columns) | 0.019 | 14 | 0.0014 | | |
| Total | 2.4 | 20 | | | |

| Number of families | 1 | | | | |
|---|---|---|---|---|---|
| Number of comparisons per family | 6 | | | | |
| Alpha | 0.05 | | | | |
| Dunnett's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.89 | 0.80 to 0.99 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml ZOA | 0.053 | -0.039 to 0.15 | No | ns | 0.4689 |
| DV-infected vs. DV+12.5 µg/ml ZOA | 0.13 | 0.041 to 0.23 | Yes | ** | 0.0036 |
| DV-infected vs. DV+25 µg/ml ZOA | 0.44 | 0.34 to 0.53 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/mlZOA | 0.81 | 0.72 to 0.90 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | 0.18 | 0.087 to 0.27 | Yes | *** | 0.0002 |

| Number of families | 1 | | | | |
|---|---|---|---|---|---|
| Number of comparisons per family | 6 | | | | |
| Alpha | 0.05 | | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value | |
| DV-infected vs. Non-infected | 0.8933 | Yes | **** | < 0.0001 | |
| DV-infected vs. DV+6.25 µg/ml ZOA | 0.05333 | No | ns | 0.3496 | |
| DV-infected vs. DV+12.5 µg/ml ZOA | 0.1333 | Yes | ** | 0.003 | |
| DV-infected vs. DV+25 µg/ml ZOA | 0.4367 | Yes | **** | < 0.0001 | |
| DV-infected vs. DV+50 µg/mlZOA | 0.81 | Yes | **** | < 0.0001 | |

| | | | | | |
|---|---|---|---|---|---|
| DV-infected vs. DV+100 µM EGCG | 0.18 | Yes | *** | 0.0002 | |

### B. ZOM vs MMP-2 expression

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.15 | 1.00 | 0.91 | 0.64 | 0.32 | 0.05 | 0.87 |
| 2 | 0.08 | 1.00 | 0.89 | 0.71 | 0.25 | 0.12 | 0.75 |
| 3 | 0.09 | 1.00 | 0.85 | 0.67 | 0.31 | 0.23 | 0.84 |
| Mean | 0.11 | 1.00 | 0.88 | 0.67 | 0.29 | 0.13 | 0.82 |
| S.D | 0.04 | 0.00 | 0.03 | 0.04 | 0.04 | 0.09 | 0.06 |
| SEM | 0.02 | 0.00 | 0.02 | 0.02 | 0.02 | 0.05 | 0.04 |

| ANOVA summary | |
|---|---|
| F | 170.7 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.9865 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 2.512 | 6 | 0.4186 | F (6, 14) = 170.7 | P < 0.0001 |
| Residual (within columns) | 0.03433 | 14 | 0.002452 | | |
| Total | 2.546 | 20 | | | |

| Number of families | 1 | | | |
|---|---|---|---|---|
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.8933 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml ZOM | 0.1167 | No | ns | 0.0526 |
| DV-infected vs. DV+12.5 µg/ml ZOM | 0.3267 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+25 µg/ml ZOM | 0.7067 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/ml ZOM | 0.8667 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | 0.1800 | Yes | ** | 0.0027 |

### C. [6]-gingerol vs MMP-2 expression

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.15 | 1.00 | 0.91 | 0.84 | 0.42 | 0.08 | 0.87 |
| 2 | 0.08 | 1.00 | 0.86 | 0.79 | 0.39 | 0.21 | 0.75 |
| 3 | 0.09 | 1.00 | 0.89 | 0.91 | 0.54 | 0.20 | 0.84 |
| Mean | 0.11 | 1.00 | 0.89 | 0.85 | 0.45 | 0.16 | 0.82 |
| S.D | 0.04 | 0.00 | 0.03 | 0.06 | 0.08 | 0.07 | 0.06 |
| SEM | 0.02 | 0.00 | 0.02 | 0.04 | 0.05 | 0.04 | 0.04 |

| ANOVA summary | |
|---|---|
| F | 290.7 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.9920 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 2.401 | 6 | 0.4001 | F (6, 14) = 290.7 | P < 0.0001 |
| Residual (within columns) | 0.01927 | 14 | 0.001376 | | |
| Total | 2.420 | 20 | | | |

## M.11 Statistical Analysis of gene expression of MMP-9 for non-infected, DV-infected and DV-infected-Treated Vero cells

### A. ZOA vs MMP-9 expression

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.07 | 1.00 | 0.96 | 0.93 | 0.84 | 0.77 | 0.71 |
| 2 | 0.05 | 1.00 | 0.98 | 0.95 | 0.79 | 0.71 | 0.77 |
| 3 | 0.03 | 1.00 | 0.95 | 0.91 | 0.83 | 0.79 | 0.85 |
| Mean | 0.05 | 1.00 | 0.96 | 0.93 | 0.82 | 0.76 | 0.78 |
| S.D | 0.02 | 0.00 | 0.02 | 0.02 | 0.03 | 0.04 | 0.07 |
| SEM | 0.01 | 0.00 | 0.01 | 0.01 | 0.02 | 0.02 | 0.04 |

| ANOVA summary | |
|---|---|
| F | 265 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.99 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 1.9 | 6 | 0.32 | F (6, 14) = 265 | P < 0.0001 |
| Residual (within columns) | 0.017 | 14 | 0.0012 | | |
| Total | 1.9 | 20 | | | |

| Number of families | 1 | | | |
|---|---|---|---|---|
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.95 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml ZOA | 0.037 | No | ns | 0.6281 |
| DV-infected vs. DV+12.5 µg/ml ZOA | 0.070 | No | ns | 0.1103 |
| DV-infected vs. DV+25 µg/ml ZOA | 0.18 | Yes | *** | 0.0001 |
| DV-infected vs. DV+50 µg/mlZOA | 0.24 | Yes | **** | < 0.0001 |

| | | | | |
|---|---|---|---|---|
| DV-infected vs. DV+100 µM EGCG | 0.22 | Yes | **** | < 0.0001 |

### B. ZOM vs MMP-9 expression

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.15 | 1.00 | 0.91 | 0.88 | 0.56 | 0.15 | 0.87 |
| 2 | 0.08 | 1.00 | 0.97 | 0.83 | 0.59 | 0.19 | 0.75 |
| 3 | 0.09 | 1.00 | 0.96 | 0.89 | 0.54 | 0.23 | 0.84 |
| Mean | 0.11 | 1.00 | 0.95 | 0.87 | 0.56 | 0.19 | 0.82 |
| S.D | 0.04 | 0.00 | 0.03 | 0.03 | 0.03 | 0.04 | 0.06 |
| SEM | 0.02 | 0.00 | 0.02 | 0.02 | 0.02 | 0.02 | 0.04 |

| ANOVA summary | |
|---|---|
| F | 291 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.99 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 2.4 | 6 | 0.40 | F (6, 14) = 291 | P < 0.0001 |
| Residual (within columns) | 0.019 | 14 | 0.0014 | | |
| Total | 2.4 | 20 | | | |

| | | | | | |
|---|---|---|---|---|---|
| Number of families | 1 | | | | |
| Number of comparisons per family | 6 | | | | |
| Alpha | 0.05 | | | | |
| Dunnetts's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.89 | 0.80 to 0.99 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml ZOA | 0.053 | -0.039 to 0.15 | No | ns | 0.4689 |
| DV-infected vs. DV+12.5 µg/ml ZOA | 0.13 | 0.041 to 0.23 | Yes | ** | 0.0036 |
| DV-infected vs. DV+25 µg/ml ZOA | 0.44 | 0.34 to 0.53 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/mlZOA | 0.81 | 0.72 to 0.90 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | 0.18 | 0.087 to 0.27 | Yes | *** | 0.0002 |

### C. [6]-gingerol vs MMP-9 expression

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.07 | 1.00 | 0.98 | 0.92 | 0.84 | 0.75 | 0.71 |
| 2 | 0.05 | 1.00 | 0.89 | 0.86 | 0.76 | 0.68 | 0.77 |
| 3 | 0.03 | 1.00 | 0.91 | 0.83 | 0.69 | 0.61 | 0.85 |
| Mean | 0.05 | 1.00 | 0.93 | 0.87 | 0.76 | 0.68 | 0.78 |
| S.D | 0.02 | 0.00 | 0.05 | 0.05 | 0.08 | 0.07 | 0.07 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| SEM | 0.01 | 0.00 | 0.03 | 0.03 | 0.04 | 0.04 | 0.04 |

| ANOVA summary | |
|---|---|
| F | 104 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.98 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 1.8 | 6 | 0.30 | F (6, 14) = 104 | P < 0.0001 |
| Residual (within columns) | 0.040 | 14 | 0.0029 | | |
| Total | 1.8 | 20 | | | |

| | | | | |
|---|---|---|---|---|
| Number of families | 1 | | | |
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.95 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml [6]-gingerol | 0.073 | No | ns | 0.3963 |
| DV-infected vs. DV+12.5 µg/ml [6]-gingerol | 0.13 | Yes | * | 0.0454 |
| DV-infected vs. DV+25 µg/ml [6]-gingerol | 0.24 | Yes | *** | 0.0005 |
| DV-infected vs. DV+50 µg/ml [6]-gingerol | 0.32 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | 0.22 | Yes | *** | 0.0009 |

## M.12 Statistical Analysis of gene expression of TIMP-1 for non-infected, DV-infected and DV-infected-Treated Vero cells

A. **ZOA vs TIMP-1 expression**

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.09 | 1.00 | 1.09 | 1.98 | 2.55 | 3.02 | 1.33 |
| 2 | 0.07 | 1.00 | 1.14 | 1.87 | 2.67 | 3.11 | 1.23 |
| 3 | 0.11 | 1.00 | 1.10 | 1.94 | 2.77 | 3.07 | 1.45 |
| Mean | 0.09 | 1.00 | 1.10 | 1.90 | 2.70 | 3.10 | 1.30 |
| S.D | 0.02 | 0.00 | 0.03 | 0.06 | 0.11 | 0.05 | 0.11 |
| SEM | 0.01 | 0.00 | 0.02 | 0.03 | 0.06 | 0.03 | 0.06 |

| ANOVA summary | |
|---|---|
| F | 728 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |

| R square | 1.0 | | | | |
|---|---|---|---|---|---|
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Treatment (between columns) | 19 | 6 | 3.2 | F (6, 14) = 728 | P < 0.0001 |
| Residual (within columns) | 0.061 | 14 | 0.0044 | | |
| Total | 19 | 20 | | | |

| Number of families | 1 | | | |
|---|---|---|---|---|
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.91 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml ZOA | -0.11 | No | ns | 0.2286 |
| DV-infected vs. DV+12.5 µg/ml ZOA | -0.93 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+25 µg/ml ZOA | -1.7 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/mlZOA | -2.1 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | -0.34 | Yes | *** | 0.0001 |

B. **ZOM vs TIMP-1 expression**

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.09 | 1 | 1.19 | 2.98 | 3.12 | 3.72 | 1.33 |
| 2 | 0.07 | 1 | 1.28 | 2.91 | 3.02 | 3.83 | 1.23 |
| 3 | 0.11 | 1 | 1.3 | 2.87 | 3.23 | 3.97 | 1.45 |
| Mean | 0.09 | 1 | 1.3 | 2.9 | 3.1 | 3.8 | 1.3 |
| S.D | 0.02 | 0 | 0.059 | 0.056 | 0.11 | 0.13 | 0.11 |
| SEM | 0.012 | 0 | 0.034 | 0.032 | 0.061 | 0.072 | 0.064 |

| ANOVA summary | | | | | |
|---|---|---|---|---|---|
| F | 848.8 | | | | |
| P value | < 0.0001 | | | | |
| P value summary | **** | | | | |
| Are differences among means statistically significant? (P < 0.05) | Yes | | | | |
| R square | 0.9973 | | | | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Treatment (between columns) | 33.32 | 6 | 5.554 | F (6, 14) = 848.8 | P < 0.0001 |
| Residual (within columns) | 0.09160 | 14 | 0.006543 | | |
| Total | 33.32 | 6 | 5.554 | F (6, 14) = 848.8 | P < 0.0001 |

| Number of families | 1 | | | |
|---|---|---|---|---|
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.9100 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml ZOM | -0.2567 | Yes | ** | 0.0079 |
| DV-infected vs. DV+12.5 µg/ml | -1.920 | Yes | **** | < 0.0001 |

| | | | | |
|---|---|---|---|---|
| ZOM | | | | |
| DV-infected vs. DV+25 µg/ml ZOM | -2.123 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/ml ZOM | -2.840 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | -0.3367 | Yes | *** | 0.0009 |

C. **[6]-gingerol vs TIMP-1 expression**

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.09 | 1.00 | 1.23 | 2.21 | 2.98 | 3.67 | 1.33 |
| 2 | 0.07 | 1.00 | 1.31 | 2.48 | 3.07 | 3.51 | 1.23 |
| 3 | 0.11 | 1.00 | 1.31 | 2.49 | 2.97 | 3.35 | 1.45 |
| Mean | 0.09 | 1.00 | 1.30 | 2.40 | 3.00 | 3.50 | 1.30 |
| S.D | 0.02 | 0.00 | 0.05 | 0.16 | 0.06 | 0.16 | 0.11 |
| SEM | 0.01 | 0.00 | 0.03 | 0.09 | 0.03 | 0.09 | 0.06 |

| ANOVA summary | |
|---|---|
| F | 448.3 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.9948 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 26.33 | 6 | 4.389 | F (6, 14) = 448.3 | P < 0.0001 |
| Residual (within columns) | 0.1371 | 14 | 0.009790 | | |
| Total | 26.47 | 20 | | | |

| Number of families | 1 | | | |
|---|---|---|---|---|
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.9100 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml [6]-gingerol | -0.2833 | Yes | * | 0.0163 |
| DV-infected vs. DV+12.5 µg/ml [6]-gingerol | -1.393 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+25 µg/ml [6]-gingerol | -2.007 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/ml [6]-gingerol | -2.510 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | -0.3367 | Yes | ** | 0.0046 |

## M.13 Statistical Analysis of gene expression of TIMP-2 for non-infected, DV-infected and DV-infected-Treated Vero cells

### A. ZOA vs TIMP-2 expression

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.10 | 1.00 | 1.10 | 1.88 | 3.86 | 4.81 | 2.24 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2 | 0.18 | 1.00 | 1.07 | 1.97 | 3.92 | 4.75 | 2.12 |
| 3 | 0.19 | 1.00 | 1.12 | 1.91 | 3.81 | 4.78 | 2.04 |
| Mean | 0.16 | 1.00 | 1.10 | 1.90 | 3.90 | 4.80 | 2.10 |
| S.D | 0.05 | 0.00 | 0.03 | 0.05 | 0.06 | 0.03 | 0.10 |
| SEM | 0.03 | 0.00 | 0.02 | 0.03 | 0.03 | 0.02 | 0.06 |

| ANOVA summary | |
|---|---|
| F | 2968 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.9992 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 48.93 | 6 | 8.155 | F (6, 14) = 2968 | P < 0.0001 |
| Residual (within columns) | 0.03847 | 14 | 0.002748 | | |
| Total | 48.97 | 20 | | | |

| | | | | |
|---|---|---|---|---|
| Number of families | 1 | | | |
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.8433 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+6.25 µg/ml ZOA | -0.09667 | No | ns | 0.1601 |
| DV-infected vs. DV+12.5 µg/ml ZOA | -0.9200 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+25 µg/ml ZOA | -2.863 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/mlZOA | -3.780 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | -1.133 | Yes | **** | < 0.0001 |

## B. ZOM vs TIMP-2 expression

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.10 | 1.00 | 2.73 | 4.17 | 5.88 | 6.27 | 2.24 |
| 2 | 0.18 | 1.00 | 2.67 | 4.53 | 5.56 | 6.89 | 2.12 |
| 3 | 0.19 | 1.00 | 2.34 | 4.26 | 5.86 | 6.94 | 2.04 |
| Mean | 0.16 | 1.00 | 2.60 | 4.30 | 5.80 | 6.70 | 2.10 |
| S.D | 0.05 | 0.00 | 0.21 | 0.19 | 0.18 | 0.37 | 0.10 |
| SEM | 0.03 | 0.00 | 0.12 | 0.11 | 0.10 | 0.22 | 0.06 |

| ANOVA summary | |
|---|---|
| F | 474.8 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.9951 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 107.1 | 6 | 17.85 | F (6, 14) = 474.8 | P < 0.0001 |
| Residual (within columns) | 0.5264 | 14 | 0.03760 | | |
| Total | 107.6 | 20 | | | |

| | | | | |
|---|---|---|---|---|
| Number of families | 1 | | | |
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.8433 | Yes | *** | 0.0006 |
| DV-infected vs. DV+6.25 µg/ml ZOM | -1.580 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+12.5 µg/ml ZOM | -3.320 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+25 µg/ml ZOM | -4.767 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/ml ZOM | -5.700 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | -1.133 | Yes | **** | < 0.0001 |

## C. **[6]-gingerol vs TIMP-2 expression**

| Trials | Non-infected | DV-infected | DV+ 6.25 µg/ml | DV+ 12.5 µg/ml | DV+ 25 µg/ml | DV+ 50 µg/ml | DV+ 100 µM EGCG |
|---|---|---|---|---|---|---|---|
| 1 | 0.1 | 1 | 1.34 | 1.83 | 4.57 | 5.02 | 2.24 |
| 2 | 0.18 | 1 | 1.48 | 1.96 | 4.29 | 5.89 | 2.12 |
| 3 | 0.19 | 1 | 1.95 | 2.03 | 3.28 | 6.01 | 2.04 |
| Mean | | | | | | | |
| S.D | | | | | | | |
| SEM | | | | | | | |

| ANOVA summary | |
|---|---|
| F | 84 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.97 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 63 | 6 | 11 | F (6, 14) = 84 | P < 0.0001 |
| Residual (within columns) | 1.8 | 14 | 0.13 | | |
| Total | 65 | 20 | | | |

| | | | | |
|---|---|---|---|---|
| Number of families | 1 | | | |
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| DV-infected vs. Non-infected | 0.8433 | Yes | * | 0.0495 |
| DV-infected vs. DV+6.25 µg/ml [6]-gingerol | -0.5900 | No | ns | 0.2286 |
| DV-infected vs. DV+12.5 µg/ml [6]-gingerol | -0.9400 | Yes | * | 0.0265 |
| DV-infected vs. DV+25 µg/ml [6]-gingerol | -3.047 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+50 µg/ml [6]-gingerol | -4.640 | Yes | **** | < 0.0001 |
| DV-infected vs. DV+100 µM EGCG | -1.133 | Yes | ** | 0.0074 |

**M.14 Multi-factor Analysis of variance of Relative Permeability of MDCK monolayer by DV infected CM following treatment with ZOM, [6]-gingerol and ZOA.**

**A.Relative Permeability of MDCK monolayer by non-infected, DV-infected and DV-infected-EDTA treated CM**

| | Relative Permeability (%) | | |
|---|---|---|---|
| | CM-Non infected cells | CM-DV infected cells | CM-DV-10mmol EDTA |
| Trial 1 (Mean) | 6.13 | 70.36 | 14.38 |
| Trial 2 (Mean) | 5.61 | 69.48 | 15.24 |
| Trial 3 (Mean) | 5.24 | 72.35 | 12.29 |
| Mean | 5.66 | 70.73 | 13.97 |
| SD | 0.44 | 1.47 | 1.52 |
| SEM | 0.26 | 0.85 | 0.88 |

| ANOVA summary | |
|---|---|
| F | 2416 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.9988 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 7526 | 2 | 3763 | F (2, 6) = 2416 | P < 0.0001 |
| Residual (within columns) | 9.347 | 6 | 1.558 | | |
| Total | 7535 | 8 | | | |

| Number of families | 1 | | | |
|---|---|---|---|---|
| Number of comparisons per family | 2 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| CM-Non infected cells vs. CM-DV infected cells | -65.07 | Yes | **** | < 0.0001 |
| CM-Non infected cells vs. CM-DV-10mmol EDTA | -8.310 | Yes | *** | 0.0003 |

**A. ZOA vs Relative permeability**

| | Relative Permeability (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Non-infected | DV-infected | DV+6.25 µg/ml ZOA | DV+12.5 µg/ml ZOA | DV+25 µg/ml ZOA | DV+50 µg/mlZOA | DV+100 µM EGCG |
| Trial 1 (Mean) | 7.62 | 100.00 | 98.96 | 89.36 | 84.23 | 52.12 | 27.57 |
| Trial 2 (Mean) | 5.27 | 100.00 | 95.34 | 82.54 | 79.92 | 67.28 | 36.13 |
| Trial 3 (Mean) | 4.68 | 100.00 | 91.53 | 94.23 | 81.37 | 72.38 | 29.63 |
| Mean | 5.90 | 100.00 | 95.00 | 89.00 | 82.00 | 64.00 | 31.00 |
| SD | 1.60 | 0.00 | 3.70 | 5.90 | 2.20 | 11.00 | 4.50 |
| SEM | 0.90 | 0.00 | 2.10 | 3.40 | 1.30 | 6.10 | 2.60 |

| ANOVA summary | |
|---|---|
| F | 143 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.98 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 22846 | 6 | 3808 | F (6, 14) = 143 | P < 0.0001 |
| Residual (within columns) | 373 | 14 | 27 | | |
| Total | 23219 | 20 | | | |

| Number of comparisons per family | 6 | | | |
|---|---|---|---|---|
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| Non-infected vs. DV-infected | -94 | Yes | **** | < 0.0001 |
| DV+6.25 µg/ml ZOA vs. DV-infected | -4.7 | No | ns | 0.7438 |
| DV+12.5 µg/ml ZOA vs. DV-infected | -11 | No | ns | 0.0767 |
| DV+25 µg/ml ZOA vs. DV-infected | -18 | Yes | ** | 0.0036 |
| DV+50 µg/mlZOA vs. DV-infected | -36 | Yes | **** | < 0.0001 |
| DV+100 µM EGCG vs. DV-infected | -69 | Yes | **** | < 0.0001 |

## B. ZOM vs Relative permeability

| | Relative Permeability (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Non-infected | DV-infected | DV+6.25 µg/ml ZOA | DV+12.5 µg/ml ZOA | DV+25 µg/ml ZOA | DV+50 µg/mlZOA | DV+100 µM EGCG |
| Trial 1 (Mean) | 7.62 | 100.00 | 80.77 | 83.27 | 42.32 | 4.77 | 27.57 |
| Trial 2 (Mean) | 5.27 | 100.00 | 92.39 | 74.26 | 49.16 | 11.83 | 36.13 |
| Trial 3 (Mean) | 4.68 | 100.00 | 87.24 | 86.64 | 37.71 | 5.37 | 29.63 |
| Mean | 5.90 | 100.00 | 87.00 | 81.00 | 43.00 | 7.30 | 31.00 |
| SD | 1.60 | 0.00 | 5.80 | 6.40 | 5.80 | 3.90 | 4.50 |
| SEM | 0.90 | 0.00 | 3.40 | 3.70 | 3.30 | 2.30 | 2.60 |

| ANOVA summary | |
|---|---|
| F | 216 |
| P value | < 0.0001 |
| P value summary | **** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.99 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 27031 | 6 | 4505 | F (6, 14) = 216 | P < 0.0001 |
| Residual (within columns) | 291 | 14 | 21 | | |
| Total | 27322 | 20 | | | |

| | | | | |
|---|---|---|---|---|
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| Non-infected vs. DV-infected | -94 | Yes | **** | < 0.0001 |
| DV+6.25 µg/ml ZOM vs. DV-infected | -13 | Yes | * | 0.0152 |
| DV+12.5 µg/ml ZOM vs. DV-infected | -19 | Yes | *** | 0.0010 |
| DV+25 µg/ml ZOM vs. DV-infected | -57 | Yes | **** | < 0.0001 |
| DV+50 µg/ml ZOM vs. DV-infected | -93 | Yes | **** | < 0.0001 |
| DV+100 µM EGCG vs. DV-infected | -69 | Yes | **** | < 0.0001 |

### C. [6]-gingerol vs Relative permeability

| | Relative Permeability (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Non-infected | DV-infected | DV+6.25 µg/ml ZOA | DV+12.5 µg/ml ZOA | DV+25 µg/ml ZOA | DV+50 µg/mlZOA | DV+100 µM EGCG |
| Trial 1 (Mean) | 7.62 | 100.00 | 92.93 | 78.83 | 56.24 | 37.52 | 27.57 |
| Trial 2 (Mean) | 5.27 | 100.00 | 98.68 | 87.66 | 51.78 | 35.67 | 36.13 |
| Trial 3 (Mean) | 4.68 | 100.00 | 94.17 | 82.42 | 63.94 | 25.46 | 29.63 |
| Mean | 5.90 | 100.00 | 95.00 | 83.00 | 57.00 | 33.00 | 31.00 |
| SD | 1.60 | 0.00 | 3.00 | 4.40 | 6.10 | 6.50 | 4.50 |
| SEM | 0.90 | 0.00 | 1.70 | 2.60 | 3.60 | 3.70 | 2.60 |

| | |
|---|---|
| ANOVA summary | 209 |
| F | < 0.0001 |
| P value | **** |
| P value summary | Yes |
| Are differences among means statistically significant? (P < 0.05) | 0.99 |
| R square | 209 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 23547 | 6 | 3925 | F (6, 14) = 209 | P < 0.0001 |
| Residual (within columns) | 263 | 14 | 19 | | |
| Total | 23810 | 20 | | | |

| | | | | |
|---|---|---|---|---|
| Number of comparisons per family | 6 | | | |
| Alpha | 0.05 | | | |
| Dunnett's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value |
| Non-infected vs. DV-infected | -94 | Yes | **** | < 0.0001 |
| DV+6.25 µg/ml [6]-gingerol vs. DV-infected | -4.7 | No | ns | 0.5993 |
| DV+12.5 µg/ml [6]-gingerol vs. DV-infected | -17 | Yes | ** | 0.0014 |
| DV+25 µg/ml [6]-gingerol vs. DV-infected | -43 | Yes | **** | < 0.0001 |
| DV+50 µg/ml [6]-gingerol vs. DV-infected | -67 | Yes | **** | < 0.0001 |
| DV+100 µM EGCG vs. DV-infected | -69 | Yes | **** | < 0.0001 |

**BINITA KOIRALA SHARMA**
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E-mail Address: binita_keshab@yahoo.com

**Personal Data:**

| | | | |
|---|---|---|---|
| Status: | Married | Name: of Spouse: | Dr. Keshab Sharma |
| Birthday: | August 9 | Name of children: | Ms. Keshbini Sharma |
| Birthplace: | Parsyang-5, Pokhara, Nepal | Religion: | Hindu |
| Language: | English, Nepali, Hindi, Filipino, Spanish | Citizenship: | Nepali |

**Educational Profile:**

B. Sc (Microbiology): Tri-Chandra Multiple College, Kathmandu, Nepal
2001-2004

M.Sc (Medical Microbiology): Tribhuvan University, Kirtipur, Nepal
2004-2006

PhD (Biological Science): University of Santo Tomas, Manila Philippines

**Masteral Thesis:**

"COMPARATIVE STUDY OF POLYMERASE CHAIN REACTION (PCR) AND LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR DIRECT DETECTION OF *Mycobacterium tuberculosis* IN SPUTUM."

**Doctoral Thesis:**

"EFFECT OF *ZINGIBER OFFICINALE* RHIZOME EXTRACTS AND [6]-GINGEROL ON THE ACTIVITY AND EXPRESSION OF MMP-2, -9 AND TIMP-1, -2 INVOLVED IN VASCULAR LEAKAGE IN AN *IN VITRO* MODEL OF DENGUE VIRUS-3 INFECTION."

**Awards Achieved:**

- **Kaski District Topper** in School Leaving Certificate Examination (S.L.C) Board Exam 1999.

**Paper Presentations:**

**Oral**:

| Title | Conference | Venue | Date |
|---|---|---|---|
| "Anti-Metalloproteinase-2, 9 (MMP-2, MMP-9) Activities of *Zingiber officinale* Rhizome Extracts and [6]-gingerol in an *In Vitro* Model of Dengue Virus (DENV-3) Infection: Implications for Vascular Permeability and Prevention of Dengue Shock" | Philippine Society of Anatomists (PSA) 3rd Quarter Regional Assembly | University of Santo Tomas, Philippines | September, 22 2012 |
| "Inhibitory Effect of *Zingiber officinale* Rosc. Extracts and [6]-gingerol on the Activity of MMP-2, MMP-9 involved in Vascular Leakage in an *In Vitro* Model of Dengue Virus Infection" | Philippine Society of Biochemistry and Molecular Biology (PSBMB) 39th Annual Convention | Fort Ilocandia Resort Hotel, Philippines | November 29-30, 2012 |

**Posters:**

| Title | Conference | Venue | Date |
|---|---|---|---|
| "*Zingiber Officinale* Rosc. Extracts and [6]-gingerol on the activity of Matrix Metalloproteinases-2, 9 Involved in Vascular Leakage in an *In Vitro* Model of Dengue Virus Infection" | 3rd National Convention of PSCB | University of Philippines, Diliman, Quezon City | September 18-19, 2012 |

## Special Skills:

Has good English communication skills
Has good teaching experiences
Uses allotted time efficiently
Has good interpersonal relationship skills
Able and willing to learn
Can work well with others
Fast learner
Computer literate

Virus Isolation and Propagation
Microbial Culture
Mycobacterial Culture
Immunoblotting
Molecular detection of pathogens (LAMP) Polymerase Chain Reaction
Agarose Gel Electrophoresis
Polyacrilamide Gel Electrophoresis
Plasmid DNA purification
Screening of drugs for antiviral activity
Screening of drugs for antimicrobial activity
Drug susceptibility Testing for Mycobactyeria
ELISA
Loop Mediated Isothermal Amplifications
DNA/RNA Extraction using QIAgen Kits
Cell Culture
qPCR / RT-PCR/ Real-Time PCR
Animal (Balb/cmice) handling

Fluorogenic Substrate Assay
Vascular permeability assay
Zymography
Basic Knowledge of Bioinformatics

Extraction of phytochemicals from medicinal plants and screen for anti-microbial activity
Range of Immunology, Microbiology and basic Virology Procedures
Induction of Matrixmetalloproteinases from mammalian cells by viral and bacterial infections
Gene Expression Profiling during Infection
Anti-viral Drug Discovery from different medicinal plant extracts
Anti-cancer Drug Discovery from different plant extracts

## Work Experience:

- Working as a **"Lecturer" for Department of Microbiology** at Manipal College of Medical Sciences, Pokhara, Nepal from March 2014 to present.
- Working as a **"Assistant Professor" for Faculty of Medical Microbiology** at Pokhara Bigyan Tatha Prabidhi Campus, Pokhara, Nepal from March 2013 to present.
- Worked as a **"Microbiology lecturer"** for Bachelor Programme of Microbiology. **Janapriya Multiple College**, Pokhara, Nepal from April 2009 to October 2009.
- Worked as **"Microbiology and Pathology Lecturer"**. **Pokhara Technical and Health Multipurpose Institute**, Pokhara, Nepal from April 2009 to October 2009.
- Worked as a **"Microbiology lecturer"** for Bachelor's and Master's Programme of Microbiology in Kantipur College of Medical Science, Kathmandu from 2nd of April, 2006 to March, 2009.
- Worked as "**Research Assistant"** on **"German Nepal Antituberculosis Project"** Kalimati from February 2006 to October 2007.
- Worked as '**Microbiologist'** and actively involved in the field of molecular biology in collaborative tuberculosis research project between **Osaka Public Perfectural Institute of Japan and Everest International Clinic and Research Center,** Kathmandu from October 2006 to November 2007.
- Volunteer in Microbiology Section of Laboratory of Pokhara Hospital and Research Center Pvt Ltd., New Road, Pokhara from 1st of June, 2004 to 10th of December, 2004.

## Seminars/ Trainings Attended:

- Basic computer course from "Council for Technical Education and Vocational Training, Tourism Training center, Phulbari, Pokhara".
  May 7- August 6, 1999.

- **Co-ordination** in the 14th Microbiology Day, jointly organized by, Nepalese Society for Microbiology ; Microbiological Students Association of Nepal; Central Department of Microbiology ,Tribhuvan University; Department of Microbiology, Tri-Chandra College, Kathmandu.
  November 27-29, 2004.

- **"Seminar on Antibiotic Resistance"** presented by Prof. Dr. David Paterson, Director, Antibiotic Management Program, University of Pittsburgh Medical Center, USA organized by ShiGan Health Foundation, Nat'I Institute of Trop Med and Public Health Research, and Nepal Assoc for Med Laboratory Science, Kathmandu, Nepal.
  July 5, 2006.

- **"Training Workshop on Bioinformatics",** organized jointly by Microbiological students association of Nepal and Central Department of Microbiology, Tribhuvan University.
  June 12-14, 2007.

- International Symposium on **"Remaining Challenges in Leprosy" organized by Mycobacterial Research Laboratory Anandaban Hospital, Kathmandu, Nepal.**
  September 5-8, 2007.

- 29th Annual ASBP Scientific Conference **"Current Trends in Systematic Biology-Applications to Teaching, Research and Extension" organized by association of systematic Biologists of the Philippines.** TARC, University of Santo Tomas, Manilla, Philippines.
  May 16-18, 2011.

- **"Field-operable Nanoparticle-based Biosensors for Global Health, Bio-defense and Food/Water Safety".** Research and Biotechnology Division, St. Luke's Medical Center, Quezon City, Philippines.
  August 22, 2011

- **3rd Quarter Regional Assembly of Philippine Society of Anatomists**, with theme **"Anatomy in Motion"** Saint Martin De Porres Building, University of Santo Tomas, City of Manila, Philippines.
  September 22, 2012.

- **"Chemical Spill Response Training"** with aims to provide basic knowledge and skills on the proper response to chemical spill incidents. Research and Biotechnology Division, St. Luke's Medical Center, Quezon City, Philippines.
  September 25, 2012.

- **3rd National Convention of the Philippine Society for Cell Biology**, College of Science Auditorium, University of the Philippines, Diliman, Quezon City, Philippines.
  October 18-19, 2012.

- Participated in the **"xCELLigence Application Training (AT) for Antiviral Assay".** Research and Biotechnology Division, St. Lukes Medical Center, Manila Philippines.
  November 28, 2012.

- **39th Annual Convention of Philippine Society of Biochemistry and Molecular Biology** with the theme **"Agricultural Biotechnology and Biodiversity: Towards Sustainable Development**", Fort Ilocandia Resort Hotel, Laoag City, Philippines.
  November 29-30, 2012.

- Participated in the **5th National Convention of the Nepalese Society for Microbiology**, Kathmandu, Nepal. November 15-16, 2014.

## Publications:

1. Koirala Sharma, B., Ramos, J.D.A (2015). 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. ***International Journal of Pharma and Biosciences*** **6**(2): 141-53.

2. Koirala Sharma, B., Klinzing, D.C., Ramos, J.D.A. (2015). Modulatory activities of *Zingiber officinale* Roscoe methanol extract on the expression and activity of MMPs and TIMPs on dengue virus infected cells. ***Asian Pacific Journal of Tropical Disease 5***(Suppl 1): S19-S26.

3. Koirala, B., Sharma, I., Kaphle H.P., Koirala Sharma, B., Sharma, K. (2015). Prevalence of Risk Factors for Non Communicable Disease in Sarangkot, Kaski, Nepal**.** ***International Journal of Health Sciences and Research*** 5(6): 456-461

4. Supram HS, Koirala B, Rani A, Gokhale S, Bhatta DR. (2015)Prevalence of Intestinal Parasitic Infections in a Tertiary Care Center at Western Nepal: Five Years Retrospective Study. ***Journal of Pharmaceutical and Biomedical Sciences*** 05(02):154-159.

## Community Service/Co-curricular Extension:

- International Lions Club of Pokhara Peace Town, District 325B1- 2nd Vice Precicent

- Nepal National Red Cross- member

**Character References:**

**Dr. John Donnie Ramos, Ph.D.**
Prof. UST Graduate School, UST; Researcher RCNS,
Dean, College of Science

**Dr. David Klinzing, Ph.D.**
Scientist – I, Research and Biotechnology Division
St. Luke's Medical Center
Presently, Deputy Director
ABI Biotech, Pte. Ltd., Singapore

I hereby certify that the above information is true and correct to the best of my knowledge and belief.

Binita Koirala Sharma