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THE ANTIVIRAL ACTIVITY OF PHENOLIC LIPIDS GROUP AGAINST DENGUE VIRUS

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ปาริชาติ กัญญาบุญ : การออกฤทธิ์ของสารกลุ่มฟีโนลิกลิพิดในการต้านการติดเชื้อไวรัสเดงกี (THE ANTIVIRAL ACTIVITY OF PHENOLIC LIPIDS GROUP AGAINST DENGUE VIRUS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. พญ. ศิวะพร บุณยทรัพยากร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. วรินทร ชวศิริ, 85 หน้า.

ไวรัสเดงกีเป็นสาเหตุทำให้เกิดปัญหาทางสาธารณสุขในระดับโลก และยังไม่มีการรักษาที่จำเพาะเจาะจง การศึกษาก่อนหน้านี้พบว่า anacardic acid saturated (C15:0) ซึ่งเป็นสารที่อยู่ในกลุ่มฟีโนลิกลิพิด สามารถยับยั้งไวรัสตับอักเสบ ซี ได้ สำหรับงานวิจัยนี้ได้ศึกษาหาความสัมพันธ์ระหว่างโครงสร้างของสารแต่ละตัวในกลุ่มฟีโนลิกลิพิดว่ามีคุณสมบัติในการยับยั้งไวรัสเดงกี โดยใช้การศึกษาในระบบเซลล์เป็นหลัก ซึ่งผลจากการศึกษาพบว่า cardol triene (C15:3) มีคุณสมบัติสามารถยับยั้งไวรัสเดงกีได้ทั้ง 4 ซีโรทัยป์ แต่ไม่สามารถยับยั้งไวรัสซิก้าสายพันธุ์ (sv0010/15) โดยมีค่าประสิทธิภาพ (EC_{50}) เท่ากับ 5.35 ± 1.16, 7.13 ± 0.72, 8.89 ± 1.77, และ 8.21 ± 0.34 ไมโครโมลาร์ ตามลำดับ cardol triene ยังแสดงค่าความเป็นพิษต่อเซลล์ (CC_{50}) ที่ดีในเซลล์ Vero และในเซลล์เป้าหมาย HepG-2, THP-1, และ HEK-293 ที่ 207.30 ± 5.24, 140.27 ± 8.44, 129.77 ± 12.08, และ 92.80 ± 3.93 ไมโครโมลาร์ ตามลำดับ การศึกษากลไกการออกฤทธิ์ของสาร พบว่า cardol triene สามารถยับยั้งไวรัสได้ทั้งในช่วงต้นและช่วงปลายของวงจรชีวิตไวรัส โดยพบว่า cardol triene สามารถยับยั้งอาร์เอ็นเอภายในเซลล์และอนุภาคไวรัสได้ ร้อยละ 87.00 ± 6.43 และ 91.73 ± 4.53 ตามลำดับ เมื่อทดสอบหลังจากที่ไวรัสจับกับตัวรับบนผิวเซลล์ไปแล้ว cardol triene สามารถยับยั้งการเกิด fusion ระหว่าง envelope ของไวรัสและ cell membrane ได้เฉพาะในไวรัสเดงกี แต่ไม่ยับยั้งในไวรัสซิก้า การศึกษากลไกที่เกี่ยวข้องพบว่า molecular targets ของสารนี้มีความเป็นไปได้ที่จะเป็น ส่วนของ kl-loops ซึ่งพบใน envelope โปรตีนของไวรัสเดงกี นอกจากนี้ cardol triene ที่ความเข้มข้น 10 และ 20 ไมโครโมลาร์ สามารถยับยั้งการเพิ่มจำนวนของ BHK-21/DENV2 replicon ได้ที่ร้อยละ 76.60 ± 7.59 และ 89.44 ± 4.02 ตามลำดับ ดังนั้นขั้นตอนการเพิ่มจำนวนของไวรัสอาจจะเป็นเป้าหมายของ cardol triene ในช่วงปลายของวงจรชีวิตไวรัส จากการศึกษาข้างต้น งานวิจัยนี้เป็นงานวิจัยแรกที่ค้นพบว่า สารกลุ่มฟีโนลิกลิพิดที่สกัดจาก CNSL เป็นสารที่มีประสิทธิภาพเหมาะสมแก่การนำไปพัฒนาเป็นยาต้านไวรัสเดงกีได้

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5987170620 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS: FLAVIVIRUS / ANTIVIRAL ACTIVITY / PHENOLIC LIPIDS

PARICHAT KANYABOON: THE ANTIVIRAL ACTIVITY OF PHENOLIC LIPIDS GROUP AGAINST DENGUE VIRUS. ADVISOR: ASST. PROF. SIWAPORN BOONYASUPPAYAKORN, M.D., Ph.D., CO-ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., 85 pp.

Dengue virus causes a global burden that specific chemotherapy has not been established in a clinical setting. A previous report suggested that anacardic acid saturated (C15:0), a member of phenolic lipids, inhibited hepatitis C virus infection. Here, we explored structure activity relationship of phenolic lipids homologues with anti-DENV properties in the cell-based system. Our results indicated that Cardol triene (C15:3) expressed DENV1-4 inhibition, but not to Zika virus (SV0010/15) with the EC_{50s} of 5.35 ± 1.16 µM, 7.13 ± 0.72 µM, 8.98 ± 1.77 µM, and 8.21 ± 0.34 µM, respectively. Also, the compound showed good CC_{50s} to Vero cells and human-derived HepG-2, THP-1, and HEK-293 cell lines at 207.30 ± 5.24 µM, 140.27 ± 8.44 µM, 129.77 ± 12.08 µM, and 92.80 ± 3.93 µM, respectively. Time of drug addition assay revealed that the compound inhibited the virus at both early and late phases post-infection. We observed that the compound interfered intracellular RNA and infectious virion at 87.00 ± 6.43 percent and 91.73 ± 4.53 percent, respectively when introduced at post-attachment. Cardol triene inhibited only dengue fusion, but not Zika virus fusion, the mechanism of action was also investigated and results showed that the molecular targets were likely kl-loops of DENV envelope protein. Moreover, the compound inhibited BHK-21/DENV2 replicon replication with 76.60 ± 7.59 percent and 89.44 ± 4.02 percent at 10 µM and 20 µM, respectively. Therefore, the replication could be the target of cardol triene at late stage. This study showed for the first time that CNSL-derived phenolic lipid had a potential for further development as anti-dengue inhibitors.

Field of Study: Medical Microbiology
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	=	American Type culture Collection
CC_{50}	=	50% Cytotoxicity concentration
Co-att	=	Co-attachment
DDW	=	Double distilled water
DENV	=	Dengue virus
DMSO	=	Dimethyl sulfoxide
EC_{50}	=	50% Efficacy concentration
EDTA	=	Ethylenediaminetetraacetic acid
FBS	=	Fetal bovine serum
Gln	=	Glutamine
h	=	Hour
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	=	Hours-post infection
kb	=	kilobase
MEM	=	Minimum essential medium
MES	=	2-(N-morpholino) ethanesulfonic acid
min	=	Minute
ml	=	Milliliter
mM	=	Milimolar
MNTD	=	Maximal non-toxic dose
M.O.I.	=	Multiplicity of infection

NS	=	Non-structural protein
°C	=	Degree Celsius
PBS	=	Phosphate buffer saline
PFU	=	Plaque forming unit
Post-att	=	Post-attachment
Pre-att	=	Pre-attachment
RT-qPCR	=	Reverse transcriptase-quantitative polymerase chain reaction
RNA	=	Ribonucleic acid
rpm	=	Revolutions per minute
SAR	=	Structure activity relationship
TOA	=	Time of drug addition
ZIKV	=	Zika virus
µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar

CHAPTER1
INTRODUCTION

Dengue infection is a major arthropod-borne global threat. The virus spreads throughout tropical regions with 390 million infected cases per year (1). Asia represented approximately 75 percent of the global burden (2), of which both Thailand and the Philippine were two of the countries with highest incidence in 2015(3). Moreover, accumulative data from 2012 to 2017 showed the mortality rate of 0.16 percent of the cases, suggesting that dengue is still a significant public health issue in Thailand (4).

Dengue virus belongs to the family *Flaviviridae,* genus *Flavivirus,* and consists of 4 species or serotype (DENV1-4). The infectious virion contains lipid envelope surrounding an icosahedral nucleocapsid and a 11 kilobases, single stranded, positive sense RNA. Envelope (E) protein is an immunogen and a major antigenic cross-reactive agent (5). All serotypes of dengue viruses are transmitted, by *Aedes aegypti* and *Aedes albopictus* mosquitoes (2). Dendritic cells (DC) are primarily infected at the local site and the virus causes systemic infection by traveling with DC into lymphatic and circulatory system. An infection with a serotype creates lifelong immunity to that particular serotype (6), but 65-70 percent of the immunity partially cross reacted to the other serotypes. When the host is subsequently infected with another serotype, the immune responses trigger the memory of the previous infection (7) creating robust but incompetent T-cells, antibodies, and pro-inflammatory cytokines. Moreover, macrophages enhanced for opsonization were infected with virus-tagged non-neutralizing antibodies resulting in facilitating viral replication inside the macrophages, thus increasing the risk of developing severe dengue (6).

Currently, the specific chemotherapy for dengue still under development but there is already a vaccine commercially available. The challenge of the vaccine design is to stimulate a highly effective response simultaneously to all serotypes (8), and not creating vaccine-induced, antibody-dependent enhancement (9). The only licensed dengue vaccine CYD-TDV Sanofi Pasteur is effective to serotype 1-4 at 54.70 percent, 43.00 percent, 71.60 percent, and 76.90 percent respectively, therefore WHO recommended only for people living in endemic area and between 9 and 45 years old (10).

Antiviral drugs have a role in treating the already infected patients with an aim to reduce viral replication, (i.e. the chance to progress to severe dengue diseases such as hemorrhagic shock, or multiple organ failure (11)). Natural products are rich source of potential antiviral inhibitors, especially those from medicinal plants. Recently, phenolic lipids were reported with a broad spectrum antiviral effect with proposed mechanisms at membrane-disturbing effect. The compounds were abundant in cashew nut shell which is an agricultural waste. This group of compounds are generally existed as mixture of saturated and three unsaturated hydrocarbon chains. Moreover, there is a previous study describing anacardic acid saturated as an anti-hepatitis C virus. Anacardic acid, one of the phenolic lipids, was reported to inhibit hepatitis C virus replication at several steps such as entry, translation, replication, and release. In this study, we aim to determine efficacies of three members of phenolic lipids consisting of anacardic acids, cardols, and cardanols including the mixture and homologs to inhibit dengue virus in cell-based system. We will explore the potential candidates for broad spectrum activity to dengue serotype 1-4 and Zika virus, as well as searching for

mechanism of drug action. We expected that this study would bring insights into further experiments on characterizing potential targets involving in viral replication.

CHAPTER2
OBJECTIVES

The objective of this study are:

1. To examine the efficacies of phenolic lipid mixture and each purified product to dengue virus serotype 2 in cell culture system.

2. To examine the broad spectrum activity of a phenolic lipid representative to inhibit dengue virus serotype 1-4 and Zika virus.

3. To explore the mechanism of drug interaction to dengue infection at cellular level.

CHAPTER3
LITERATURE REVIEW

3.1 Virology

Dengue virus belongs to the family *Flaviviridae,* genus *Flavivirus,* and closely related to Japanese Encephalitis Virus (JEV), West Nile Virus (WNV), Zika virus (ZIKV), Yellow fever virus (YFV), and Hepatitis C virus (HCV) (12). Flaviviruses have had an important impact on human well-being over the centuries. Many flaviviruses (but not Hepatitis C virus) are transmitted by mosquitoes or ticks (13). Dengue virus (DENV) consists of 4 distinct serotypes (DENV1-4). All four serotypes are known to cause the full spectrum of disease. The virion contains lipid at 20 percent by weight (14). The virion possesses a spherical nucleocapsid core and an icosahedral-like envelope with a diameter around 50 nm (15). The DENV genome is approximately 11 kb long, with a 5′ cap (m7G5′ppp5′A) at the 5′ end, but lacks a polyadenylate tail (16). This genomic RNA also serves as an mRNA containing a single open reading frame to translate into a large polyprotein, which is subsequently cleaved into three structural proteins (capsid protein C, membrane protein M and envelope protein E) and seven non- structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (17). Non-structural proteins play various roles in controlling and regulating the intracellular processes to facilitate the viral replication.

3.2 Viral life cycle

The *Flavivirus* replication cycle starts at envelope (E) protein binding to the receptors on the cell membrane including heparin sulfate

(18), DC-SIGN (19), mannose receptor (20), TIM/TAM receptor (21), Hsp70, Hsp90 (22), GRP78/p protein (23), or PtdEth CD300a (24), and entering the target cells such as monocytes, macrophages, dendritic cells, mast cells, hepatocytes and endothelial cells (25) via receptor-mediated endocytosis (26). The viral membrane fuses with host endosomal membrane under acidic condition (pH~5-6) thus releasing the RNA genome into the cytoplasm. Translation takes place by utilizing the host ribosomal machineries associated with the endoplasmic reticulum (ER). Following cleavage of the polyprotein by cellular and viral (NS2B/NS3) proteases. The viral replication complex (RNA, NS5, nonstructural proteins and cellular factor) forms inside the intracellular membranes. Evidences showed that viral replication occurs in ER-derived vesicular structures (27). Negative sense RNA was synthesized from the genome and serves as template for the positive sense RNA replicates. The virion assembly occurs in the lumen by budding into the endoplasmic reticulum (ER) and rearranges its membrane and envelope proteins while traveling through the golgi complex. Finally, mature infectious virus are released from the cells by exocytosis (28, 29) (Figure 3.1)

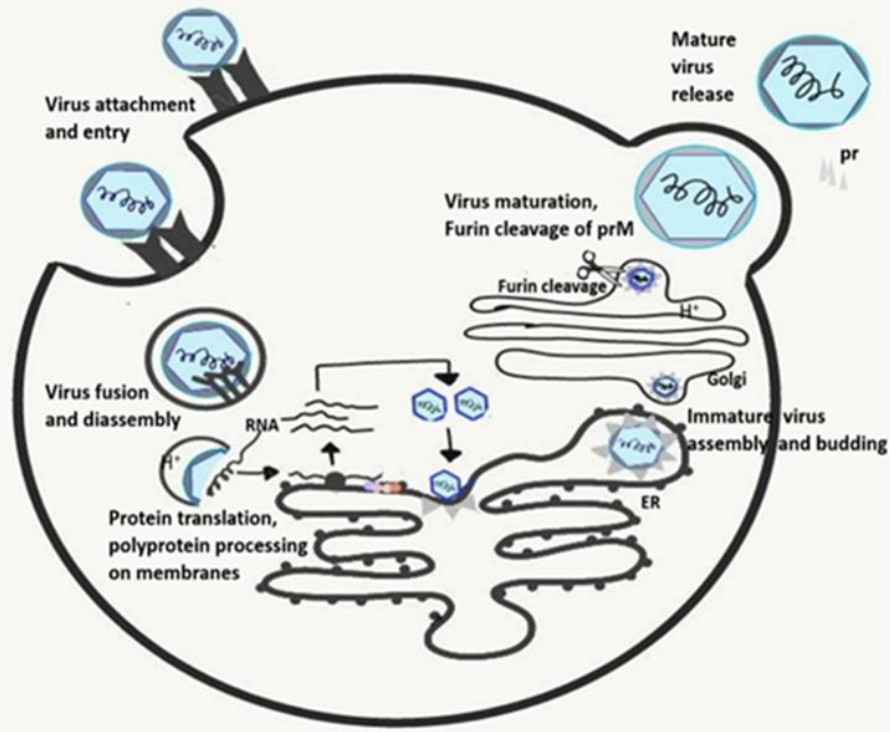


Figure 3.1 Flavivirus replication pathway.

Antiviral drugs for dengue targeted both structural and non-structural proteins (Figure 3.2) involving in many steps in viral replication such as entry (30), fusion (31), assembly, maturation (32). To start with, the capsid protein is being explored as a drug target (33). The capsid protein is the first viral protein to be synthesized during translation and is shown to be integrated in the membrane of the endoplasmic reticulum by the hydrophobic anchor and projecting into the cytoplasm (34). This protein assembled to form an icosahedral capsid for packaging the RNA genome.

The envelope protein E consists of three domains: domain I (N-terminal, structurally central), domain II (containing the hydrophobic fusion peptide), and domain III (receptor-binding). Protein E also represents the first point of contact between the virus and the host cell and

is able to interact with several cellular proteins and carbohydrate molecules (35)

NS1 is a glycoprotein that is translocated into the lumen of the endoplasmic reticulum and secreted from the cell, NS1 showed a stable hydrophobic homodimer that interacts with membrane (36, 37), or may expressed on the surface of infected cells and serve as a target for human antibody response to DENV infections. This protein is involved in viral RNA replication (38, 39) and may be also involved in assembly of the viral replication complex and its localization to cytoplasmic membranes (40).

NS2A protein serves as a component of the viral replication complex that functions in RNA synthesis and virion assembly (41).

NS2B-NS3 protease, the viral NS3 protease is required for processing of the viral polyprotein and viral replication. NS2B is an integral membrane protein that serves as a cofactor that play role in regulation of the catalytic activity of NS3 protein (42-44). The multifunctional enzyme NS2B-NS3 (32, 33) is a major target in many drug discovery projects.

NS4A and NS4B are highly hydrophobic integral membrane protein, NS4A play role in induction of membrane alterations required for virus replication, while NS4B is involved in direct interactions with the NS3 protein enhances the helicase activity of NS3, supporting viral RNA replication (45-47).

NS5 protein (Bi-functional: viral methyltransferase polymerase) is the largest and the most conserved DENV protein. This protein is also a major target for antiviral drug discoveries (34). This bi-functional enzyme consist an N-terminal methyltransferase and C-terminal RNA-dependent RNA-polymerase domain (48, 49).

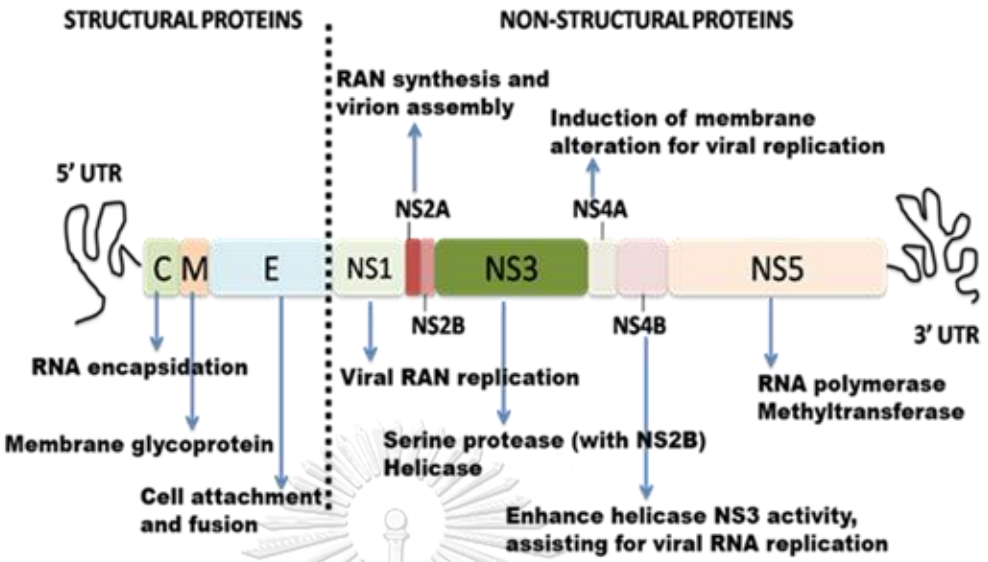


Figure 3.2 Structures of dengue virus proteins.

3.3 Epidemiology and pathology

Dengue is endemic in more than 100 countries in southeast Asia, the Americas, the western Pacific, Africa, and the eastern Mediterranean regions (3). The dengue virus have a transmission cycle that requires invertebrate vectors (mosquitoes *Aedes aegypti* and *Aedes albopictus*) (50). Also, travelers play important role in global epidemiology, causing the spread from continent to continent crossing the anatomical barriers.

Dengue virus causes a wide range of diseases in human from the acute febrile illness dengue fever (DF) to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (WHO, 2009). DF symptomatic are characterized by fever, headache, retro-orbital pain, myalgia, arthralgia, and rash. DHF is pointed by increased vascular permeability (plasma leakage), thrombocytopenia, and hemorrhagic manifestations, DSS grow when fluid leakage into the interstitial spaces

results in shock (3), that without treatment may lead to death. Recently, dengue mortality and burden for Global Burden of Disease study in 2013 showed there are 60 million symptomatic dengue infections per year, resulting in about 10,000 deaths (51). Moreover, Bureau of the Vector-borne Diseases, Department of Disease Control, Ministry of Public Health reported accumulative data from 2012 to 2018 showed the mortality rates at 0.13 percent of the case (4).

3.4 Treatment

3.4.1 Dengue vaccine development

Currently, there is a commercially available vaccine named Dengvaxia (CYD-TDV) developed by Sanofi Pasteur. CYD-TDV is the chimeric yellow fever-dengue vaccine (CYD) that employs the yellow fever virus 17D strain as the replication backbone (52). CYD-TDV is effective to serotype 1-4 at 54.7 percent, 43.0 percent, 71.6 percent, and 76.9 percent respectively, overall protective efficacy of 65.6 percent but was substantially more effective against severe dengue and dengue hemorrhagic. WHO recommended only for people living in endemic area and between 9 and 45 years old (10). Several other vaccines have been developed using a variety of technological advancements including live attenuated chimeric dengue vaccines, TV003/TV005 (whole virus DENV 1-3 and recombinant DENV2 in DENV4 backbone) by NIAID and Butantan Institute (53) and DENVax (whole virus DENV2 and recombinant DENV1/3/4 in DENV2 backbone) by Takeda (54). DEN-80E tetravalent E protein subunit V180 vaccine by Merck (54). These vaccines have been shown to be immunogenic in animals and also safe and immunogenic in humans. However, these vaccines are just to progress to clinical trial phase II-III to set their protective efficacy of dengue virus (55).

3.4.2 Dengue drug development

Antiviral drugs is aimed to reduce viral replication in the febrile state patients with an objective to alleviate the severity, (e.g. plasma leakage, hemorrhage, shock, or multiple organ failure (11)). Studying the molecular mechanism of dengue replication including the specific functions of each DENV protein has led to the identification of new anti-viral targets. In addition, with the help of novel technologies like *in silico* screening, ligand-based design, high-throughput viral replication, and enzymatic assays increasing the number of newly identified leads against dengue infection (56-58). The potential inhibitors reported for anti-dengue activity were listed (Table 3.1). Moreover, there are 5 compounds undergoing clinical trials (Table 3.2) (59). Notably, Ivermectin from Phase II clinical trial in Thailand reduced NS1 levels in serum and body temperature with high dose (55).

Table 3.1 The development of anti-dengue drug.

Targets	Compound	Mode of action	EC_{50} µM	CC_{50} µM	References
E-protein	1662G07	E stem and E trimer interaction	16.90	>100	Lim *et al*., 2013 (11)
E-protein	GNF-2 (8b)	Interact with E protein	IC_{90} DENV1=4 DENV2=5 DENV3=10 DENV4=40	CC_{90} BHK21-DENV2 =60	Clark *et al*., 2016 (60)
M-protein	MLH40 peptide	Block DENV-2 attachment to cells	24.00-31.00	ND	Panya *et al*., 2015 (61)
C-protein	Capsid inhibitor ST-148	C protein	0.016	>100	Lim *et al*., 2013 (11)
NS2B-NS3 protease	Tetrapeptide aldehyde Bz-nKRR-H	Bound to NS2B-NS3 protease	5.00	BHK-21 =>300 HuH7 =55.00	Li *et al.*, 2014 (62)
NS2B-NS3 protease	CID 54681617 CID 54692801 CID 54715399	Inhibit NS2B-NS3 protease	61.50±4.60 14.90±2.90 11.80±0.20	162.40±0.90 58.60±3.00 42.10±1.60	Montalvo *et al*., 2016 (63)
NS3 protease	Bromocriptine	Inhibit translation and/or replication	0.80-1.60	53.60	Fumihiro *et al*., 2016 (64)
NS2B-NS3 protease	Mefenamic acid, doxycycline	Against dengue virus replication	32.00±1.00 40.00±3.00	150.00±5.00 125.00±4.00	Rothan *et al*., 2013 (65)
NS4B	2-oxopiperazine derivatives-4	NS4B	All four serotypes < 0.10	>50.00	Kounde *et al*., 2017 (66)
NS5 protein	BG-323	MTase GTP pocket	30.80±1.30	184.00±4.20	Lim *et al*., 2015 (67)
NS5 protein	4-HPR	-	2.50	13.50	Lim *et al*., 2015 (67)
NS5 protein	Ivermectin	-	3.00	7.00-10.00	Lim *et al*., 2015 (67)
NS5 RdRp	Compound 12 and 21 (thaiazolidinone-thaidiazole scaffold)	Inhibit DENV-2 NS5 RdRp	2.30 2.10	ND	Manvar *et al*., 2015 (68)

ND=Not determined

Table 3.2 The therapeutic trials listed on anti-dengue drug.

Compound	**Rationale**	**Subject Characteristics**	**Results**	**Reference**
Chloroquine	Antimalarial drug, fusion inhibitor	Age, >18 y: trial size 307, Vietnam	No change in viremia and NS1 antigenemia	Tricou *et al*., 2010 (69)
Prednisolone	Anti-inflammatory properties	Age, 5-20 y : trial size 225, Vietnam	No change in virological profile	Tam *et al*., 2012 (70)
Balapiravir	NS5 nucleoside inhibitor	Age, 18-65 y: trial size 64, Vietnam	No change in virological and immunological profile	Nguyen *et al*., 2013 (71)
Celgosivir	ER-associated and glucosidase inhibitor	Age, 21-65 y: trial size 50, Singapore	No statistically significant reduction of viral load	Low *et al*., 2014 (72)
Lovastatin	Cholesterol synthesis inhibitor, required for viral RNA assembly	Age, >18 y: trial size 300, Vietnam	No evidence of effect on any manifestations	Whitehorn *et al*., 2016 (73)

3.5 Phenolic lipids and derivatives

Natural products are rich source of potential antiviral inhibitors, especially those from traditional medicinal plants. The potential inhibitors reported for anti-dengue activity were listed (Table 3.3) (74). Phenolic lipids isolated from cashew nutshell liquid (CNSL) composed of long aliphatic chains and phenolic rings, including simple phenols, polyphenols and their derivatives (75). The major determinant of phenolic lipid species are defined by the head group of CNSL into anacardic acid, cardol, and cardanol (Table 3.4). The constituent of CNSL differ depending on the method of extraction. For example, when CNSL is extracted by cold temperature- or solvent-dependent method, the components are approximately 74.10-77.40 percent anacardic acid, 15.00-20.10 percent cardol, 1.20-9.20 percent cardanol, and a few percentage of other phenols (76). In contrast, the heating method leads to decarboxylation of anacardic acid to cardanol therefore the components are approximately 60.00-65.00 percent cardanol, 15.00-20.00 percent cardol, 10.00 percent polymeric material (77). The constituent of CNSL are mixtures of four types of C15 fatty acids consisting of 5.00-8.00 percent saturated, 48.00-49.00 percent monoene, 16.00-17.00 percent diene, and 29.00-30.00 percent triene (76) homologues. Previously, phenolic lipids were reported with biological effects such as anti-parasitic, anti-cancer, anti-fungal, anti-microbial, anti-inflammatory, and anti-oxidant (Table 3.5). Phenolic lipids present very remarkable amphiphilic properties and can interaction with membrane structures and the hydrophobic domains of protein. Therefore the ability of these compounds to inhibit microbial, fungal, and parasitic growths likely depend on their interaction with protein and/or on their membrane-disturbing properties (78) (Table 3.5). Moreover, the diversity of chemical

structure in phenolic lipids may account for the diverse bioactivities. For example, the inhibitory effects of anacardic acid, one of the phenolic lipids, on hepatitis C virus proposed mechanisms at entry, translation, replication, and release (79). Furthermore, cardol was a promising anti-cancer chemotherapy for anti-proliferative/cytotoxic properties with IC_{50} of 13.17±0.76 µM (80).

Table 3.3 Natural product with reported for develop anti-dengue drugs.

Species	**Common name**	**Compound isolated**	**EC_{50} (µM)**	**CC_{50} (µM)**	**References**
Flagellaria indica	Whip Vine	Galactan	12.50	312.50	AbdKadir *et al*., 2013 (74)
Houttuynia cordata	Pak Kan Thong	Hyperoside	0.19-1.70	312.50	AbdKadir *et al*., 2013 (74)
Rhizophora apiculata	Bakau	-	12.5 0	625.00	AbdKadir *et al*., 2013 (74)
Distictella elongate (Vahl)	-	-	11.10±1.60	ND	Simoes *et al*., 2011 (81)
Phyllanthus spp.	-	-	Inhibit >90%	MNTD 250	Lee *et al*., 2013 (82)
Nephelium lappaceum	Rambutan	Geraniin	1.75	Not toxic to vero cells upto 2 mM	Admad *et al*., 2017 (83)

ND=Not determined

Table 3.4 The molecular structure of anacardic acid, cardanol, and cardol from CNSL.

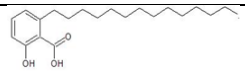
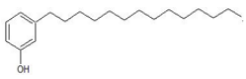
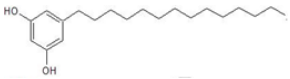
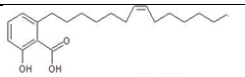
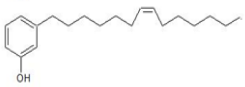
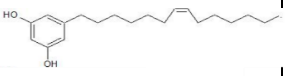
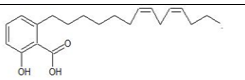
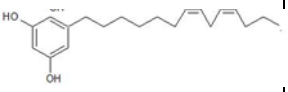
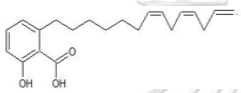
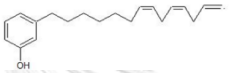
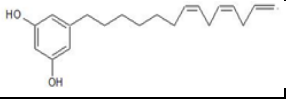
	Anacarcic acid	Cardanol	Cardol
N=0; Saturated			
N=1; Monoene			
N=2; Diene			
N=3; Triene			

Table 3.5 Biological activities of phenolic lipid and derivatives

Subclasses	**compound**	**Biological activities**	**Results**	**References**
Anacardic acid	*Gingko biloba* ($C_{22}H_{36}O_3$)	Inhibit entry, translation replication, and secretion with no effects on cell viability	IC_{50}=7.50 µM	Hundt *et al*., 2015 (79)
Cardanol	Thai *Apis mellifera* Propolis	Interaction with proteins or on their membrane-disturbing properties	Antibacterial activities -IC_{50} 0.58 uM for *E. coli* -IC_{50} 2.27 uM for *Paenibacillus larvae*	Boonsai *et al*., 2014 (84)
Anacardic acid	Cashew nut cell liquid (6-pentadecylsalicylic acid)	Prevent *M. oryzae* from infection by induce apoptosis-like cell death	Antifungal activities (*Magnaporthe oryzae*) -at 75.00 uM, complete inhibition of spore germination -at 5 uM, 62.00%±5.00 of conidia showed membrane constriction -at 50 uM, 72.00% of hyphae showed	Muzaffar *et al*., 2016 (85)

Subclasses	compound	Biological activities	Results	References
			membrane constriction	
Cardol	*Trigona incisa* propolis	Induced apoptosis in the SW620 human colorectal cancer cell line	Antiproliferative activity IC_{50} 13.17±0.76 uM	Kustiawan *et al*., 2017 (80)
Cardol	*Trigona incisa* propolis	Cytotoxicity and apoptosis in cancer cell lines	Antiproliferative/ cytotoxic activity - IC_{50} 2.23±0.22 uM (Hep-G2) - IC_{50} 2.55±0.18 uM (Chaco) - IC_{50} 13.46±0.10 uM (BT474) - IC_{50} 14.18±0.76 uM (SW620) - IC_{50} 19.06±0.39 uM (KATO-III)	Kustiawan *et al*., 2015 (86)
Cardanol	3-pentadecylphenol (PDP)	Interact with the structure of the lipids bilayers of DPPC liposome	Shown PDP/DPPC isotropic aggregates and lower gauche populations in the hydrophobic chain	Cieslik-Boczula and Koll, 2009 (87)
Cardol, anacardic acid, and methyl cardol	-	Interact with the structure of the lipids bilayers	Cardol affected the properties of the hydrophobic region but anacardic acid and methylcardol were altered the properties of the subsurface part	Stasiuk and Kozuber, 2008 (88)
Cardanol		Preincorporated into a 1-palmitoyl-2-oleylphosphatydi-choline liposome bilayer	Had a stabilizing effect on the liposome	De Maria *et al*., 2005 (89)

CHAPTER4
MATERIALS AND MEDTHODS

4.1 Cell cultures

4.1.1 Vero cells

Vero cells (ATCC®CCL-81), derived from kidney of adult African green monkey were maintained in Medium 199, Earle' salts (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 I.U./ml penicillin (Bio Basic Inc., Canada), and 100 µg/ml streptomycin (Bio Basic Inc., Canada), 10 mM HEPES (4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid) (Sigma Aldrich. USA) and 10% $NaHCO_3$ (Sigma Aldrich. USA) at 37°C in humidified air with 5% CO_2. Cells were sub-cultured with growth medium (see in appendix B) and using pre-warmed 0.05% trypsin-EDTA (see in appendix B) every three days and were maintained until they were senescent in culture.

4.1.2 LLC/MK2 cells

LLC/MK2 cells (ATCC®CCL-7), derived from kidney of adult rhesus monkey were maintained in minimal essential medium (MEM) (Gibco, USA) supplemented with 10% FBS, 100 I.U./ml penicillin, and 100 µg/ml streptomycin, 10 mM HEPES and 10% $NaHCO_3$ at 37°C in humidified air with 5% CO_2.. Cells were sub-cultured with growth medium (see in appendix B) every three days and were maintained until they were senescent in culture.

4.1.3 C6/36 cells

C6/36 cells (ATCC®CRL-1660), a continuous mosquito cell line was derived from a larva of *Aedes albopictus*, were maintained in MEM medium supplemented with 10% FBS, 100 I.U./ml penicillin, and 100 µg/ml streptomycin, 10 mM HEPES and 10% $NaHCO_3$ at 28°C. Cells were sub-cultured for removed the old medium by re-suspension in growth medium (see in appendix B).

4.1.4 BHK-DENV2 replicon cells

BHK-DENV2 replicon cells, a stable hamster kidney (BHK) cell line expressing the DENV2 Rluc reporter replicon, were maintained in MEM medium supplemented with 10% FBS, and 0.3 mg/ml G418 (Bio Basic Inc., Canada), 10 mM HEPES and 10% $NaHCO_3$ at 37°C in humidified air with 5% CO_2. Cells were sub-cultured with growth medium (see in appendix B) with split-ratio of 1:10 and were maintained until they were senescent in culture.

4.2 Virus stock preparation

4.2.1 Dengue virus propagation

Dengue virus serotype 1-4 were reference laboratory strains containing DENV1 (16007), DENV2 (New Guinea C strain), DENV3 (16562), were a gift from Assoc. Prof. Padet Siriyasatien, M.D., Ph.D., and DENV4 (C0036) was a gift from Prof. Kiat Ruxrungtham, M.D., and Assist. Prof. Chutitorn Ketloy, Ph.D., Chulalongkorn University. DENV were propagated in Vero cell line with maintenance medium 199 (see in appendix B) at 37°C in humidified air with 5% CO_2. The monolayer cells in T25 cell culture flask were infected with DENV and rocking every 15

minutes for 1 h before incubating until the cytopathic effect (CPE) of infected cells were observed under microscope after three day post infection (dpi). Then, supernatant was used for other infections. The process was repeated for three passages to high viral titer. Next, supernatant was collected by centrifuged to remove cell debris at 1,500 rpm, 4°C, 5 min and FBS was added to the final concentration of 20 % before storing as aliquots at -70°C. The viral titer was quantified by plaque titration assay.

4.2.2 Zika virus propagation

Zika virus (SV0010/15) was a gift from Armed Forces Research Institute of Medical Sciences (AFRIMS), and Department of Disease Control, Ministry of Public Health, Thailand. Zika virus was propagated in C6/36 cells with maintenance medium MEM (see in appendix B) at 28 °C. The process as previously described. The viral titer was quantified by plaque titration assay.

4.3 Compounds separation and purification

All phenolic lipids were received from Assist. Prof. Warinthorn Chavasiri, Ph.D., Chulalongkorn University. The compounds were extracted and separated from cashew nut shell liquid (CNSL). Briefly, CNSL (50g) was dissolved in 5% aqueous MeOH (300 mL) (Sigma Aldrich. USA) and $Ca(OH)_2$ (50g) (Sigma Aldrich. USA) was added in portions under stirring before the reaction mixture was stirred at 50°C for 5 h. The reaction was monitored by thin layer chromatography (TLC) (performed on aluminum sheet pre-coated with silica gel, Merck's Kieselgel 60 PF_{254}) for the absence of CNSL. After completion of the reaction, the precipitated calcium anacardate was filtered and washed with

MeOH and the precipitate was dried under vacuum evaporator. Calcium anacardate was suspended in water, acidified with conc. HCL (Sigma Aldrich. USA) and stirred for 1 h. The resultant solution was extracted with EtOAc (Sigma Aldrich. USA). The organic layer was washed with water, dried over anhydrous Na_2SO_4 (Sigma Aldrich. USA), and concentrated under reduced pressure to yield anacardic acid (42.1 g, 84% yield). The filtrate left from the filtration of calcium anacardate was evaporated under reduced pressure and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain crude product which was purified by silica column eluting with hexane/EtOAc to afford cardanol (1.1g, 2% yield) and cardol (319.1 mg, 0.1% yield). The constituents of mixture compounds were separated by semi-preparative HPLC. Cardanol derivatives and cardol derivatives were systhesized by hydrogenation. All compounds were stored as solid powder at room temperature. The stock solution compounds were prepared in dimethyl sulfoxide (DMSO) (Merck, USA) to 50 mM final concentration and stored as aliquots at -20°C until use.

4.4 Plaque titration of virus

The 24-well plates plaque titration assay for DENV and Zika virus. Briefly, LLC/MK2 ($5x10^4$) cells were seeded into each well of 24-well plates and incubated overnight at 37°C in humidified air with 5% CO_2. The supernatant or viral stock was 10-fold serially diluted in maintenance medium before infecting cells at 100 µl/well. Then, the plate was incubated at 37°C in humidified air with 5% CO_2 with gentle rocking every 15 min for 1 h. Next, cells were washed with 1X PBS and semisolid overlayer medium, 0.8% gum tragacanth culture medium (see in appendix B) was

added. The experiment was done in duplicate and maintenance medium was used as mock infection. The plate was incubated until plaque was observed under microscope. Cells were fix and stained for 1 h with 10% formaldehyde (Carlo Erra, Italy), 5% isopropanol (Merck, Germany), and 1% crystal violet (Merck, Germany) (see in appendix B). The number of plaque forming units PFU/ml was determined manually and calculated with formula.

PFU/ml = plaque no. per dilution x Dilution factor x 10

4.5 Primary screening of phenolic lipid compounds

To explore the efficacies of anacardic acid, cardol, and cardanol mixtures against DENV2, Vero ($5x10^4$) cells were seeded into each well of 24-well plates and incubated overnight at 37°C with 5% CO_2. The cells were infected with DENV2 (M.O.I. of 0.1) with 10 µM and 25 µM concentration of compounds in 1% DMSO. 1% DMSO was used as a mock treatment with 100% infectivity rate. After that cells were incubated at 37 °C for 1 h cells were washed with PBS and incubated with compounds at designated concentrations. The supernatants were collected at 3 days post infection for plaque titration assays to select the active compounds (cut off=>90% viral inhibition). The experiment was tested by Thanaphon Saelee, Department of Biology, Faculty of Science, Chulalongkorn University.

To examine the toxicity of the mixture compounds from primary screening. Vero (10^4) Cells were seeded into each well of 96-well plates and incubated overnight at 37°C with 5% CO_2. Compounds were added at the same concentrations as in the primary screening, 1% DMSO as control

(100% cells viability). Cells were incubated for 48 h and 72 h before cell viability was accessed using CellTiler 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, USA) according to manufacturer's protocol. The plate was incubated at 37°C for 4 h and read by spectrophotometer at A_{450} nm with microplate reader model: VICTORTM X3 (PerkinElmer, USA). And results were calculated to present cell death. The experiment was tested by Thanaphon Saelee, Department of Biology, Faculty of Science, Chulalongkorn University.

4.6 Cytotoxic concentration study (CC_{50})

Vero (10^4) cells were seeded into each well of 96-well plates and incubated overnight at 37°C. Phenolic lipid mixtures and their homologue were serially diluted in DMSO to the final concentrations as follows; 25, 50, 100, 200, 300, 400, and 500 µM. Each concentration was performed in quadruplicates. 1% DMSO was used as a mock treatment referring to 100% cell viability. Cells were incubated for 48 h before cell viability was accessed using CellTiler 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, USA) according to manufacturer's protocol. The plate was incubated at 37°C for 4 h and read by spectrophotometer at A_{450} nm with microplate reader model: VICTORTM X3 (PerkinElmer, USA). Cytotoxic concentrations were calculated from non-linear regression analysis and the results were reported as means and standard error mean (SEM) from three independent experiments, unless indicated otherwise. The experiment was tested by Thanaphon Saelee, Department of Biology, Faculty of Science, Chulalongkorn University.

4.7 Effective concentration study (EC_{50})

Vero ($5x10^4$) cells were seeded into each well of 24-well plates and incubated as previously described before infected with DENV2 at the M.O.I. of 0.1 for 1 h with gentle rocking every 15 min. Phenolic lipid mixtures and their homologue were serially diluted in DMSO to the final concentrations as follows; 0, 0.5, 1, 1.5, 2, 2.5, 5, 7.5, 10, 25, and 50 µM. DMSO at the concentration of 1% was used as a mock treatment referring to 100% infection. Infected cells were treated with the designated compounds during and post infection. Supernatants were collected at 3 days after infection for analysis of virion production by plaque titration. Effective concentrations of the compounds were calculated from non-linear regression analysis and the results were reported as means and standard error mean (SEM) from three independent experiments. The therapeutic index (TI) was calculated from a ration of CC_{50}/EC_{50}.

4.8 Plaque titration assays

The 96-well plaque titration assay was adopted for determining the efficacy of the compound for viral inhibition (90). Briefly, the supernatant was 10-fold serially diluted in the culture medium and sampled 50 µl into each well of 96-well plate. LLC/MK2 ($2.5x10^5$) cells were prepared in 50 µl before gently mixed into each well. Each dilution was performed in triplicates. The plate was incubated at 37°C under 5% CO_2 for 3 h before addition of semisolid overlayer medium, 0.8% gum tragacanth culture medium (see in appendix B) 100 µl. The plate was incubated for 5 to 7 days. Cells were fixed and stained with 10% formaldehyde and 1% crystal violet, respectively. Plaques were counted and reported in plaque forming units (PFU/ml). Each experiment was done in triplicate and reported as means

and standard error mean (SEM) for further EC_{50} analysis. The number of plaque forming units (PFU/ml.) was determined manually and calculated with formula.

PFU/ml = plaque no. per dilution x Dilution factor x 20

4.9 Time of drug addition study (TOA) (91, 92)

TOA assay was designed to preliminary determine the mechanism of compound affected the DENV life cycle, the results from TOA assay used to verify the functional impairment of that particular step. Briefly, Vero ($5x10^4$) cells were seeded in each well of 24-well plates and incubated overnight. Cardol triene was prepared in DMSO and added to the final concentrations of 10 µM and 20 µM to DENV2 infected Vero cells (M.O.I. of 0.1) with independent experiments. The concentration of 10 µM of cardol triene were added at before (-1 h), during (0 h) and after infection (2, 4, 6, 8, 10, 12, 24, 48, and 72). As the 20 µM of cardol triene were added at various time points by dividing into two experiments; early time points at during (0), and after (1, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h) infections. The late time points were also accessed at (0, 12, 14, 16, 18, 20, 22, 24, 36, 48, 60, and 72 h) after infections. DMSO alone was added to the infected cells as a mock treatment. Plates were incubated for 3 days before collection of cell lysates and supernatants to determine viral RNA by RT-qPCR and virion production by plaque titration, respectively. The results were reported from three independent experiments.

4.10 Viral RNA extraction and RT-qPCR

The intracellular viral RNAs were extracted from cell lysates by TRIzol reagent (Invitrogen, Life, Grand Island, USA) according to the manufacturer's protocol. Total RNAs were stored at -70 °C until use. To determine the reduction of viral genome in experiments, total RNAs were quantified by Nanodrop (EppendorfBio Photometer D30, NY, USA). The RT-qPCR were performed using 1x Power SYBRGreen PCR Master Mix, 400 nM each of C-protein primer (93) or 400 nM of NS1 primer (94) (see in appendix C) and 0.1 µg of total RNA with a Step-OnePlus Real-Time PCR System (ABI7500, California, USA). The reactions were cycled at 48°C 30 min and 95°C 10 min, followed by 45 cycles of 95°C for 20s (denaturation), 55°C for 30s (annealing), 72°C for 30s (extension). Results were collected in cycle threshold (Ct) and analyzed by absolute quantification. Each sample was analyzed in triplicated. The results were reported from three independent experiments.

4.11 Anti-attachment study (95)

Vero ($5x10^4$) cells were seeded and inoculated in 24-well plates as previously described. Cells were then adsorbed by DENV2 (M.O.I. of 1) diluted in maintenance medium at 4°C for 1 h with continuously gentle rocking. Cardol triene at 10 µM was added to DENV2 virus preparation for 1 h before adsorption (pre-attachment), during adsorption (co-attachment), and after adsorption plus three washing with cold PBS to remove external viruses (post-attachment). Cells were incubated at 37 °C, under 5% CO_2 for 3 days before collection of cell lysates and supernatants to determine viral RNA by RT-qPCR and virion production by plaque

titration, respectively. DMSO-treated were used as no-inhibition control. The results were reported from three independent experiments.

4.12 Fusion inhibition study (96-98)

C6/36 ($2x10^5$) cells were seeded into 24-well plates and incubated at 28°C before infected with DENV2 and ZIKV (M.O.I. of 1) for 1 h with gentle rocking. Cardol triene at 10 µM were introduced to the DENV2 and ZIKV infected cells after infection. DMSO-treated cells was used as no inhibition control and 4G2-treated cells was used as a 100% inhibition control. Plates were incubate at 28°C for 2 days before addition 0.5 M 2-(N-morpholino) ethanesulfonic acid (MES) (pH 5.0) (Sigma Aldrich, USA) until fused cells were observed. Picture were taken using an Eclipse TS100 Inverted Routine Microscope (Nikon, New York, USA). The results were reported from three independent experiments.

4.13 Replicon inhibition study

BHK-21 ($5x10^4$) cells expressing DENV-2 replicon (BHK-21/DENV2) (91) were seeded in 24-well plates and incubated at 37°C under 5% CO_2 for 1 day. Cardol triene at 10 µM and 20 µM were introduced to treated cells. DMSO-treated cells was used as the no-inhibitor control (0% inhibition) and used the ribavirin (TargetMol, USA) as the reference compound. Cells were incubated at 37°C for 3 days before collection of cell lysates to quantified DENV2 replicon by RT-qPCR. Data were reported as percent inhibition compared with ribavirin. The results were reported from three independent experiments.

4.14 Cell morphology

Vero ($5x10^4$) cells were seeded and inoculated in 24-well plates as previously described. Cells were infected with DENV2 (M.O.I. of 1 and 5) for 1 h with gentle rocking. Cardol mixture and cardol triene at 10 µM were introduced to the DENV2 infected cells during and after infection. DMSO, 10 µM cardol mixture, and 10 µM cardol triene alone were used as no virus control. The cell morphology was observed after 2, 24, and 48 h incubation. Picture were taken using an Eclipse TS100 Inverted Routine Microscope (Nikon, New York, USA).

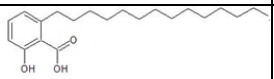
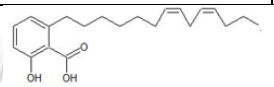
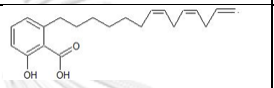
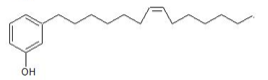
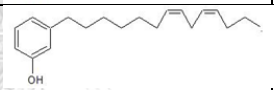
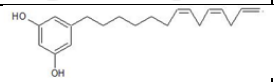
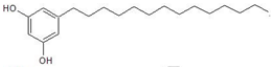
CHAPTER5
RESULTS

5.1 Compound separation and purification results

Phenolic lipids were extracted from CNSL and purified by the ^{1}H NMR ($CDCl_3$) spectroscopy. The products were initially characterized using the species of hydrophilic heads into; anacardic acid, cardanol, and cardol, with constituted of 84, 2, and 0.1 percent, respectively. Next, each species were further separated into pure homologues of saturated (C15:0), monoene (C15:1), diene (C15:2), and triene (C15:3) using semi-preparative HPLC. The characteristics and signals of all compounds were shown as follows Table 5.1

In addition, the saturated homologues (C15:0) of cardanol and cardol were synthesized from hydrogenation reaction. The characteristic signals were shown as follows Table 5.1

Table 5.1 The data of phenolic lipid from CNSL after separation and purification.

No.	Name	structure	MW	Content/ Yield
1	Anacardic acid C15:0 (6-pentadecylsalicylic acid)		348	18% of total anacardic acids
2	Anacardic acid C15:1 (6-[8(Z)-pentadecenyl]salicylic acid)		346	31% of total anacardic acids
3	Anacardic acid C15:2 (6-[8(Z), 11(Z)-pentadecadienyl]salicylic acid)		344	26% of total anacardic acids
4	Anacardic acid C15:3 (6-[8(Z), 11(Z), 14-pentadecatrienyl] salicylic acid)		342	25% of total anacardic acids
5	Cardanol C15:1 (3-[8(Z) pentadecenyl] phenol)		302	31% of total cadanols
6	Cardanol C15:2 (3-[8(Z), 11(Z)-pentadecadienyl] phenol)		300	46% of total cadanols
7	Cardanol C15:3 (3-[8(Z), 11(Z), 14-pentadecatrienyl] phenol)		298	23% of total cadanols
8	Cardanol C15:0 (3-pentadecylphenol)		304	75% yield Synthesized by hydrogenation
9	Cardol C15:2 (5-[8(Z), 11(Z)-pentadecadienyl] resorcinol)		316	73% of total cadols
10	Cardol C15:3 (5-[8(Z), 11(Z), 14-pentadecatrienyl] resorcinol)		314	27% of total cadols
11	Cardol C15:0 (5-pentadecylresorcinol)		320	83% yield Synthesized by hydrogenation

5.2 Initial screening of compounds with DENV-2 and cytotoxicity in Vero cells

A previous report suggested that anacardic acid mixture extracted from leaves of *Gingko biloba* inhibited many steps of hepatitis C virus replication cycle (79). Hepatitis C virus is one of the members of the family *Flaviviridae*, therefore, it is speculated that the inhibitory effect of anacardic acids and their close relatives would also be seen in dengue viruses. We extended our study towards the three head groups extracted from CNSL including anacardic acid, cardanol, and cardol in order to explore the possible relationship between the head group structures and the activity to inhibit dengue virus cellular replication. Briefly, anacardic acid, cardanol and cardol mixtures were co-incubated with DENV2 infected Vero cells (M.O.I. of 0.1) at 10 µM and 25 µM for 72 h and the viral inhibition was quantified by plaque titration. Our preliminary data suggested that DENV2 were inhibited at 97.95, 65.30, and 95.91 percent when incubated with anacardic acid, cardanol and cardol at 10 µM, respectively; and more than 99.00 percent at 25 µM (Fig. 5.1a). Similar effect was observed in DENV2 infected LLC/MK2 cells that were incubated for 120 h at 65.00, 97.40, and more than 99.00 percent when incubated with anacardic acid, cardanol and cardol at 10 µM, respectively; and more than 99.00 percent at 25 µM (Saelee, T., unpublished). Moreover, the viability of Vero cells were accessed in the presence of each compound at 10 and 25 µM for 48 and 72 h (Fig 5.1b) and cytotoxicity results were less than 10 percent in all experimental conditions. Taken together, our preliminary data showed the compounds inhibited DENV2 infectivity similar to that of HCV previously reported (79). Therefore, we plan to study further purified and synthesized all phenolic compounds into

homologue and characterized the efficacies and cytotoxicities of the infected cells.

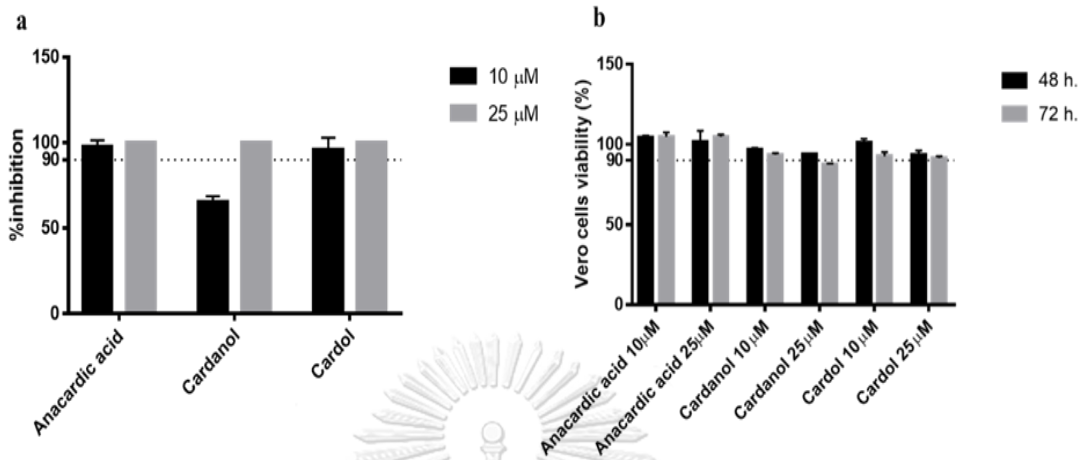


Figure 5.1 (a) Percentage of screening results in Vero cells at 72 h. (b) Toxicity of the compounds in Vero cells at 48 h and 72 h.

5.3 Cytotoxicity and efficacy study of phenolic lipid.

Each compound mixture were further purified and synthesized into saturated (C15:0), monoene (C15:1), diene (C15:2), and triene (C15:3) as previously described. Toxicities were first to be analyzed regarding the safety concern. Vero cells were treated with the compound at the following concentrations; 25, 50, 100, 200, 300, 400, and 500 μM, for 48 h before the cell viability was accessed using MTS reagents. Results were calculated to percent viability and CC_{50} were analyzed using non-linear regression curve. Interestingly, triene compounds, anacardic and cardol, strikingly showed higher CC_{50s} of 115.13 ± 14.12, and 207.30 ± 5.24 μM (Table 5.2), when compared with other homologues of the same head groups. In addition, the cell viability declined in consistent with decreasing double bonds of the tail, and reached the lowest CC_{50s} at saturation (C15:0). Cardanol triene was insoluble in DMSO, therefore the compound was excluded from analysis.

Regarding the table 5.2, cardol triene yielded the highest CC_{50} value at 207.30 ± 5.24 µM in Vero cells. This finding was verified using the observation of cell morphology under DENV2-infected (M.O.I. of 1 and 5) and 10 µM cardol triene treatment for 2, 24, and 48 h (Fig. 5.2-5.4). Moreover, we further explored the CC_{50} of cardol triene in other cell lines to verify its cytoprotective character. CC_{50s} of three human-derived cell lines (HepG2, THP-1, and HEK-293) co-incubated with cardol triene and cardol mixture (73 percent diene and 27 percent triene) were reported (Fig. 5.5) (Saelee, T, unpublished). From the results, the triene compound was apparently less cytotoxic than the mixture at about 30-50 µM in all cell types.

Next, we studied the efficacies (EC_{50}) of the phenolic compounds against DENV2 infectivity in Vero cells. Briefly, compounds at 10 different concentrations were added to DENV2 infected Vero cells (M.O.I. of 0.1) during and post infection and the viral inhibition was determine by plaque titration. Efficacies of anacardic acids series varied among homologues so that the structure-activity relationship was inconclusive. However, we observed that EC_{50s} of saturated homologue and the mixture were unusually similar due to the fact that the anacardic acid saturated constituted of 18 percent of the total mixture, plus the other components were all contributed to higher EC_{50s}. It is possible that the viral inhibition and cellular response might be predominately influenced by anacardic acid saturated regardless of the percent constituent. Noted that anacardic acids were major components of CNSL extracts where none of the homologues was synthesized in the laboratory. Therefore, obtaining the adequate amount for further experiments was an advantage of this group.

Also, cardanol mixture and homologues were studied for efficacies of DENV2 inhibition. Naturally derived cardanol mixture was a combination of monoene, diene, and triene compounds, where the cardanol saturated was a product of hydrogenation. Overall, efficacy of cardanols were less potent than anacardics and cardols. One of the homologues, cardanol triene was insoluble in DMSO; therefore, the efficacy and cytotoxicity were unable to obtain in this setting. Efficacy of cardanol mixture fell between the efficacies of the saturated and monoene, suggesting that both homologues contributed to the viral inhibition. Interestingly, hydrogenation of cardanol potentiated the efficacy as the EC_{50} went down to 5.89±2.83 µM. However, cardanols were the most cytotoxic of all, thus resulted in the least TI values. The group were then excluded from further investigations.

Last, cardol mixture and homologues were analyzed for their efficacies using the DENV2 infected cells previously described. Cardol mixture was the most potent DENV2 inhibitor beyond any homologue with the EC_{50} of 3.24± 0.51 µM, and therapeutic index (TI) (Table 5.2) of 18.67. In other words, a synergistic effect was found in CNSL-derived cardol mixture consisting of 73 percent diene and 27 percent triene homologues. Analysis of the homologue showed that increasing the number of double bonds in the tail structure resulted in increasing the viral inhibition. In other words, cardol triene was the most and cardol saturated was the least effective compound to inhibit DENV2 infectivity in Vero cells. Considering the TI values, cardol triene homologue showed the broadest TI values at 29.07, suggesting strong candidates for further characterization. From this results, cardol triene was selected as a pure homologue for subsequent mechanism a drug action study.

Moreover, we explored whether cardol triene would exhibit broad spectrum anti-dengue and Zika activities. Efficacies against DENV1 (16007), DENV3 (16562), DENV4 (c0036), and ZIKV (sv0010/15) (Table 5.3) suggested that cardol triene similarly inhibited all dengue viruses but not Zika virus. Investigating for drug target could start from the discrepancies between mechanism of dengue and Zika virus replication inside the cells, as well as between the two viral proteins.

Table 5.2 Effective concentrations (EC_{50}), cytotoxic concentration (CC_{50}), and Therapeutic index (TI) of phenolic lipids.

compounds	EC_{50} (μM)	CC_{50} (μM)	TI
Anacardic acid mixture	**4.82 ± 1.71**	**58.80 ± 2.91**	**12.19**
Anacardic acid saturated	4.31 ± 1.17	66.33 ± 1.58	15.39
Anacardic acid monoene	12.59 ± 0.84	74.00 ± 0.85	5.88
Anacardic acid diene	Not inhibited	112.17 ± 8.57	-
Anacardic acid triene	7.48 ± 2.14	115.13 ± 14.12	15.38
Cardanol mixture	**11.06 ± 0.40**	**46.86 ± 2.94**	**4.23**
Cardanol saturated	5.89 ± 2.83	43.51 ± 1.10	7.39
Cardanol monoene	7.65 ± 2.58	98.70 ± 3.16	12.90
Cardanol diene	Not inhibited	159.40 ± 7.41	-
Cardanol triene*	-	-	-
Cardol mixture	**3.24 ± 0.51**	**60.51 ± 4.94**	**18.67**
Cardol saturated	12.72 ± 0.67	58.75 ± 0.43	4.62
Cardol diene	11.90 ± 0.82	71.66 ± 5.27	6.02
Cardol triene	7.13 ± 0.72	207.30 ± 5.24	29.07

* Insoluble.

The TI values represent the ratios of CC_{50}/EC_{50}. Data represent means ± standard error mean (SEM) of at least two independent experiments.

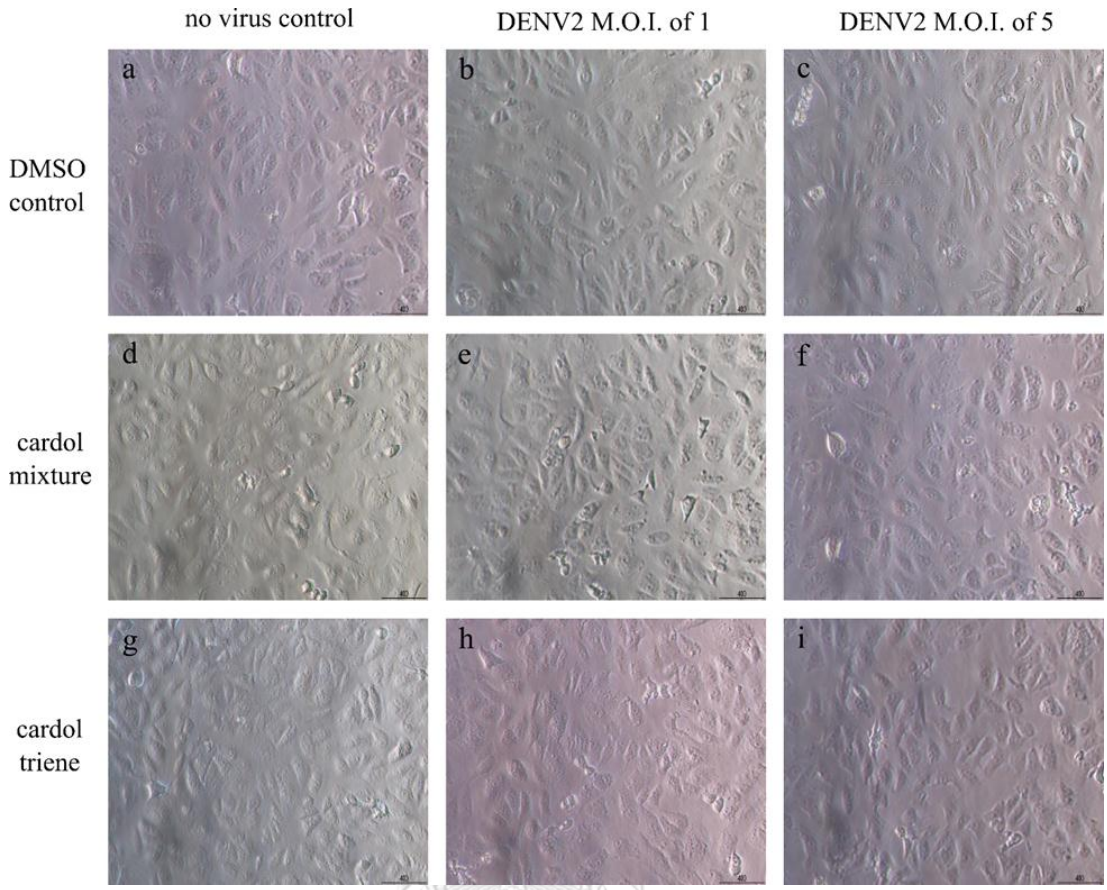


Figure 5.2 Morphology of Vero cells under DENV2 infected M.O.I. of 1 and 5 were treated with 10 µM cardol mixture and cardol triene at 2 h. Images are at 400x magnification.

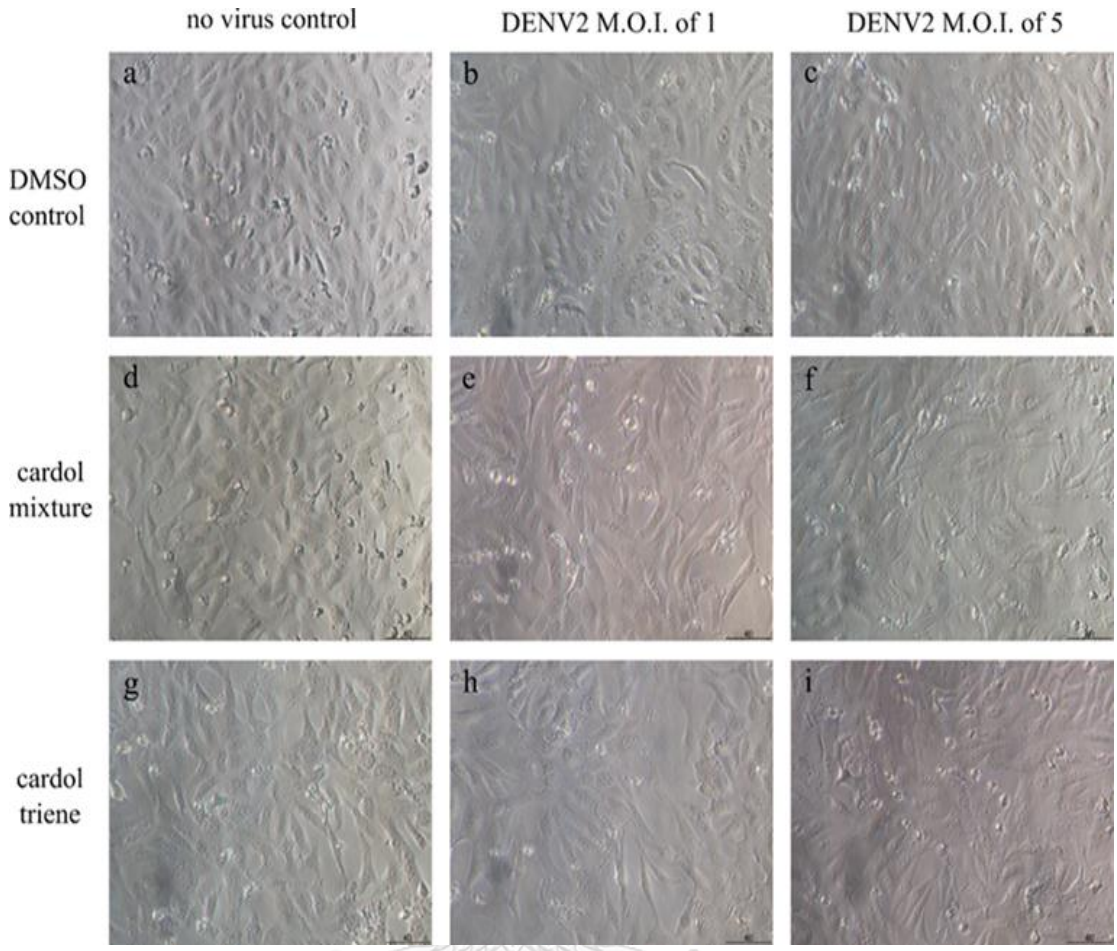


Figure 5.3 Morphology of Vero cells under DENV2 infected M.O.I. of 1 and 5 were treated with 10 µM cardol mixture and cardol triene at 24 h. Images are at 400x magnification.

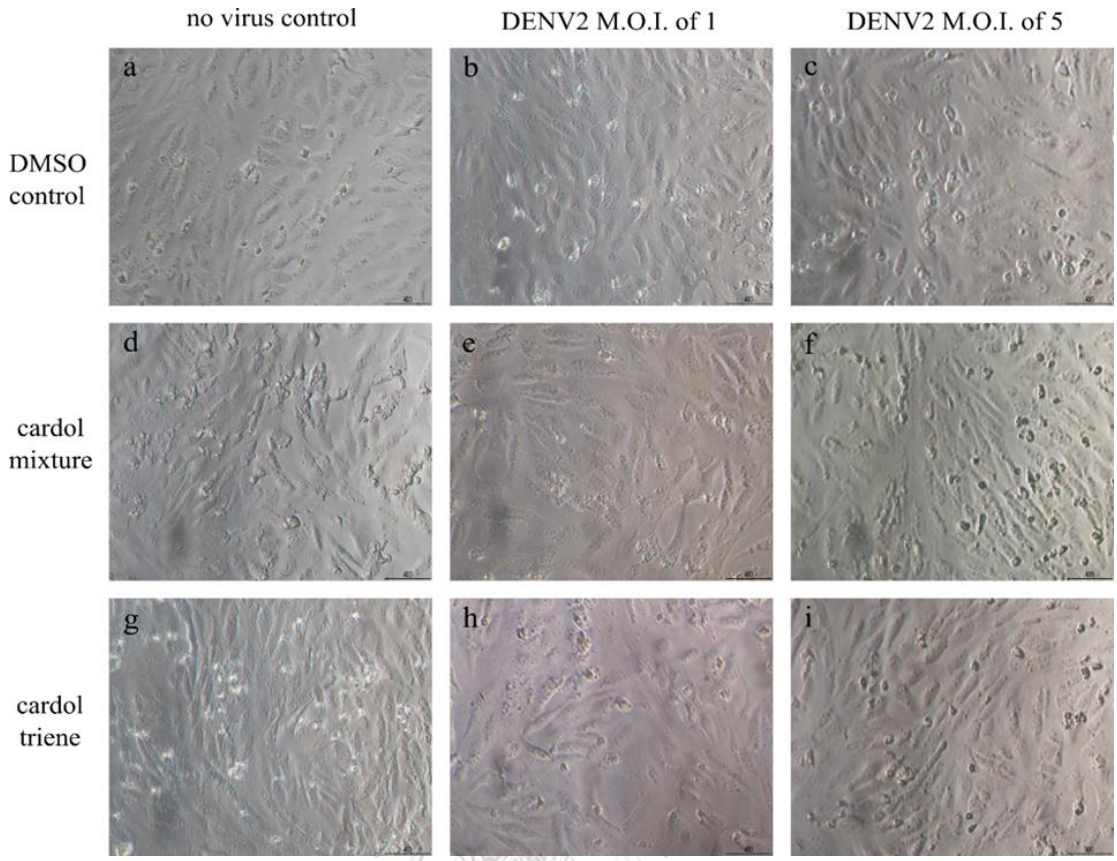


Figure 5.4 Morphology of Vero cells under DENV2 infected M.O.I. of 1 and 5 were treated with 10 µM cardol mixture and cardol triene at 48 h. Images are at 400x magnification.

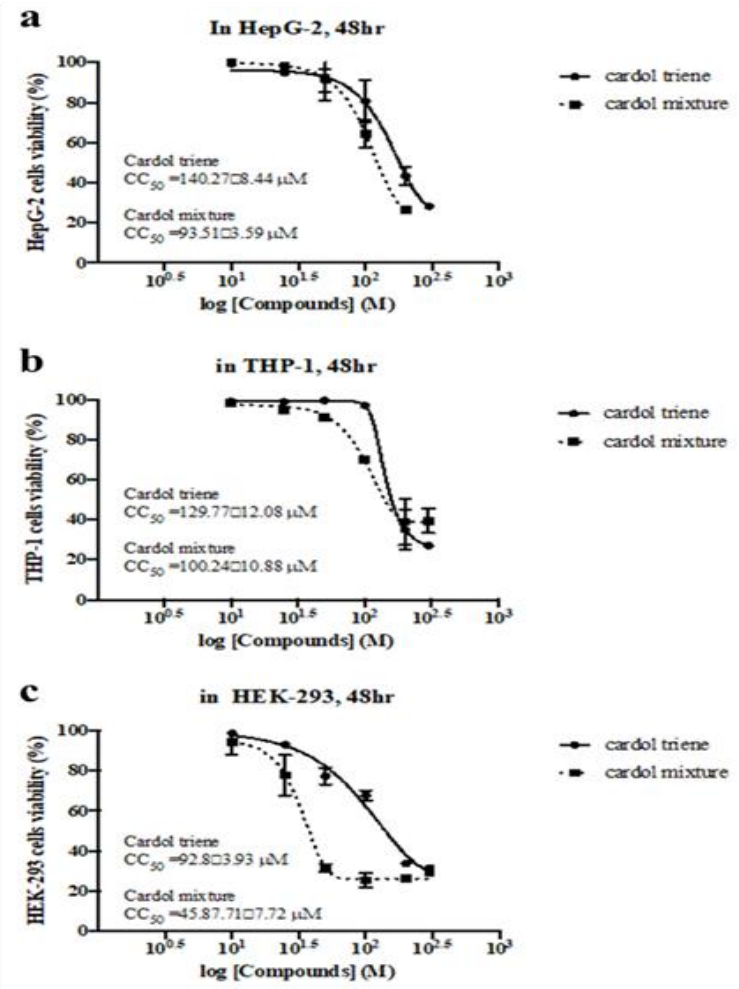


Figure 5.5 Cytotoxic concentration (CC_{50}) of cardol triene and cardol mixture when treated in (a) HepG2 cells, (b) THP-1 cells, and (c) HEK-293 cells. Data represent means ± standard error mean (SEM) of three independent experiments.

Table 5.3 Effective concentrations (EC_{50}) and Therapeutic index (TI) of cardol triene with DENV 1, 3, 4 and Zika virus.

Virus	EC_{50} (µM)	TI
DENV1	5.35±1.16	38.77
DENV3	8.98±1.77	23.08
DENV4	8.21±0.34	25.24
ZIKV	Not inhibited	-

Data represent means ± standard error mean (SEM) of three independent experiments.

5.4 Mechanism of action study

5.4.1 Initial screening mechanism: Time of drug addition

Time of drug addition assay is a standard method to primarily identify the possible target of a newly characterized compound (99-101). Based on the previous efficacy results, cardol triene was chosen as a representative of the CNSL-derived phenolic lipids. Cardol triene was prepared in DMSO and added to the final concentrations of 10 µM and 20 µM to DENV2 infected Vero cells (M.O.I. of 0.1). The compound was added to the cells at different time points. DMSO alone was added to the infected cells as a mock treatment. After 72 h incubation, cell lysates and supernatants were collected for analysis of intracellular RNAs and infectious virions by RT-qPCR and plaque titration, respectively. Results showed that intracellular RNAs and infectious virions (Fig. 5.6, 5.7) decreased immediately at 1 h and persisted until 48 h after infections suggesting that the compound inhibited the virus at both early and late phases post-infection. The inhibitory effect was also dose-dependent as 20 µM of the compound inhibited more strongly than 10 µM to DENV2 infected Vero cells. Noted that the EC_{50} of cardol triene was 7.13 ± 0.72

μM; therefore, the viral replication should be suppressed beyond 50 percent by plaque titration under the treatment of cardol triene at both concentrations. From this results, we hypothesized that the phenolic compounds could bind to multiple targets at both early and late stages of the virus life cycle.

Moreover, a computational screening was done in parallel using similarity search, bioactivity analysis and interactome mapping (courtesy of Kowit Hengphasartporn, Ph.D. candidate). Potential targets were identified and grouped into 31 protein families. Among the direct hits, a nonstructural protein-5 (NS5) was the only flaviviral protein to be identified. This result supported the TOA finding that viral replication was one of the potential targets of cardol triene and other CNSL-derived phenolic lipids.

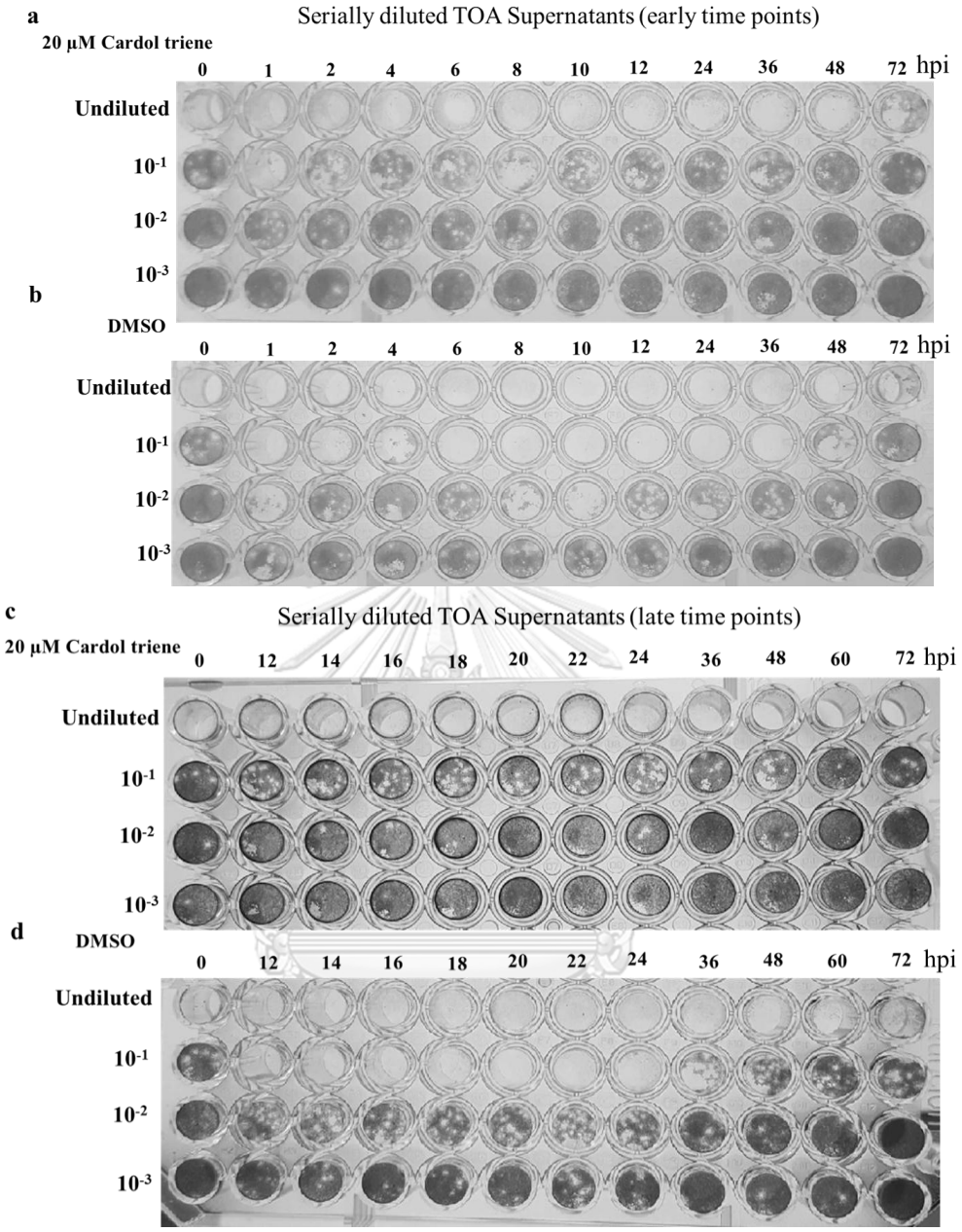


Figure 5.6 Plaque formations from TOA supernatants at (a, b) early and (c, d) late time points. Cells were infected with DENV2 at a M.O.I. of 0.1. The compound was added to the cells at different time points after infection (hpi). DMSO alone was added to the infected cells as a mock treatment. After 72 h incubation, cell lysates and supernatants were collected for analysis of intracellular RNAs and infectious virions by RT--qPCR and plaque titration, respectively

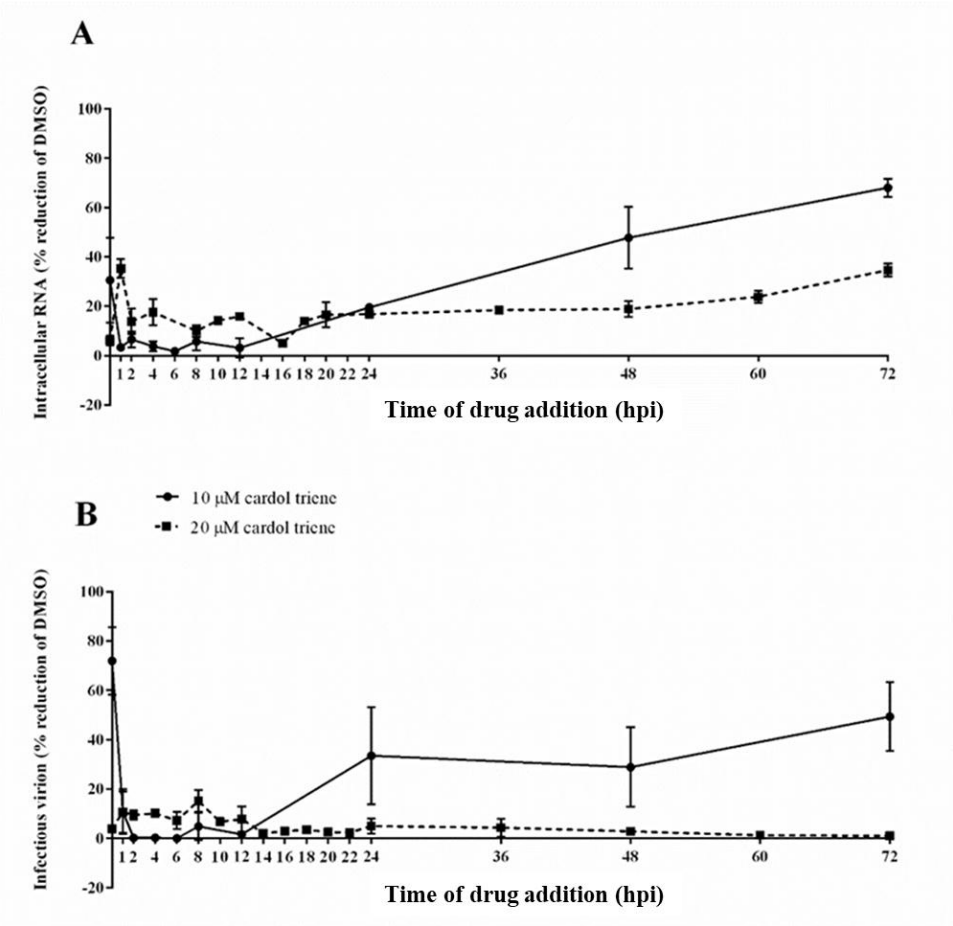


Figure 5.7 The intracellular RNAs (A) and infectious virions (B) from TOA at early and late time points were treated with 10 and 20 μM of cardol triene. Data were represent of three independent experiments.

5.4.2 Investigating the possible targets of the early steps

Molecular docking by CDOCKER revealed that cardol triene and other CNSL-derived phenolic lipids stably bound to kl-loops that were found exclusively in DENV E, not in Zika E protein (Fig. 5.8a-c) (courtesy of Kowit Hengphasartporn, Ph.D. candidate). The inhibition of viral attachment was studied using cardol triene at 10 µM co-incubated with DENV2 before, during, and after infection to Vero cells (Fig. 5.9 attachment inhibition diagram). Since dengue envelope protein was the key factor to receptor-binding, endocytosis, and fusion steps of virus life cycle, we asked the question whether the compound binding would maximally inhibit DENV envelope at which step. Results showed that the major inhibition was observed at post-attachment with interferences of intracellular RNA and infectious virion at 87.00 ± 6.43 percent and 91.73 ± 4.53 percent (Figure 5.10) respectively. This level of inhibition resembles that of EC_{50}s experiment where 10 µM cardol triene inhibited the viral progeny at 99.93 ± 0.01 percent with no significant difference. Partial inhibition was also noticed in compound-virus pre-incubation and co-incubation. Taken together, the compound most likely bound to kl-loop of envelope protein and inhibited its conformational change to trimerization, that would subsequently trigger pH-dependent fusion.

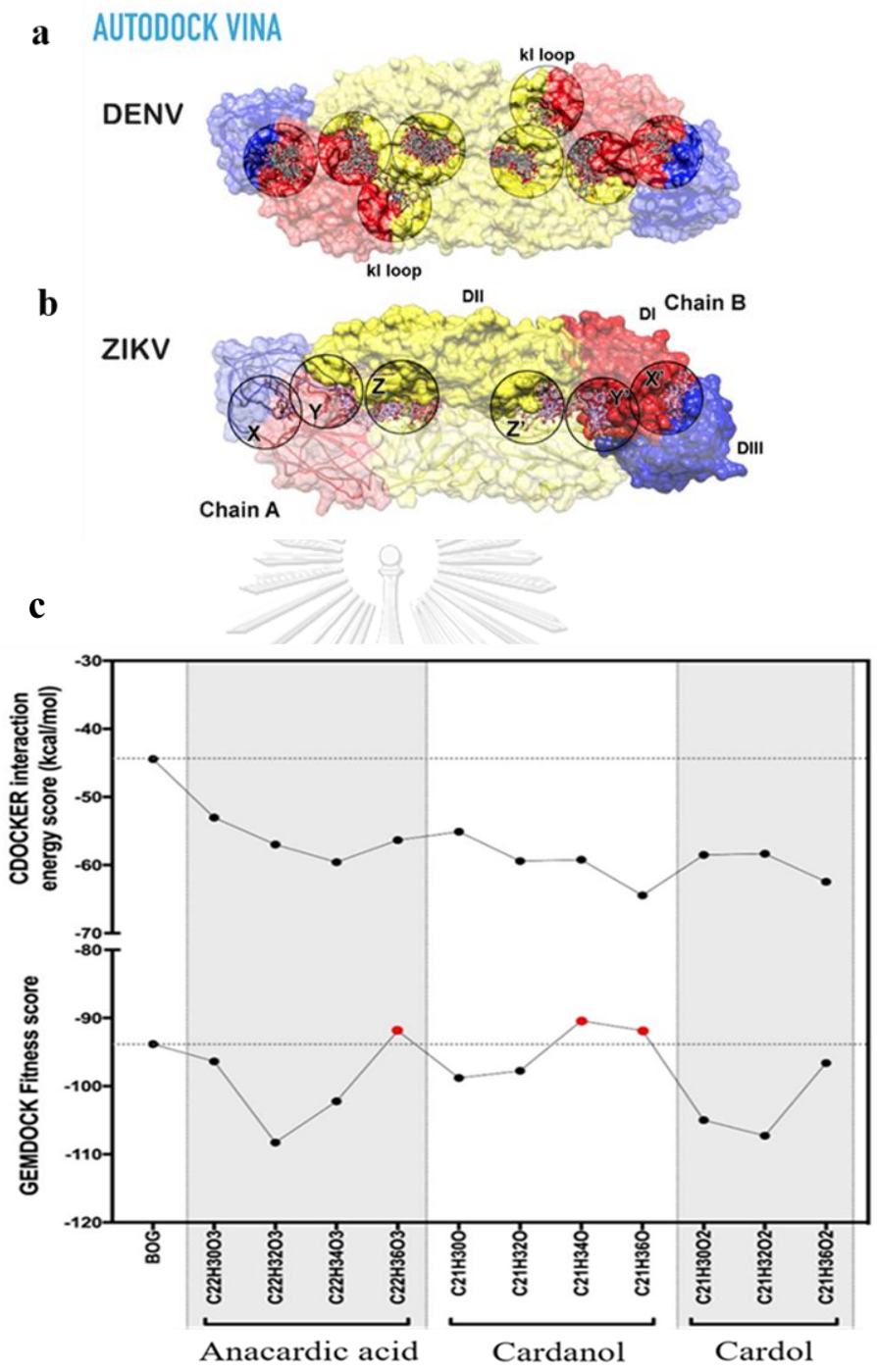


Figure 5.8 a) Crystal structure of DENV E protein with kl-loop, b) a crystal structure of Zika virus E protein without kl-loop, c) the binding energy and fitness score between series of phenolic lipid and kl-loop of DENV E protein using two molecular docking methods. (courtesy of Kowit Hengphasartporn)

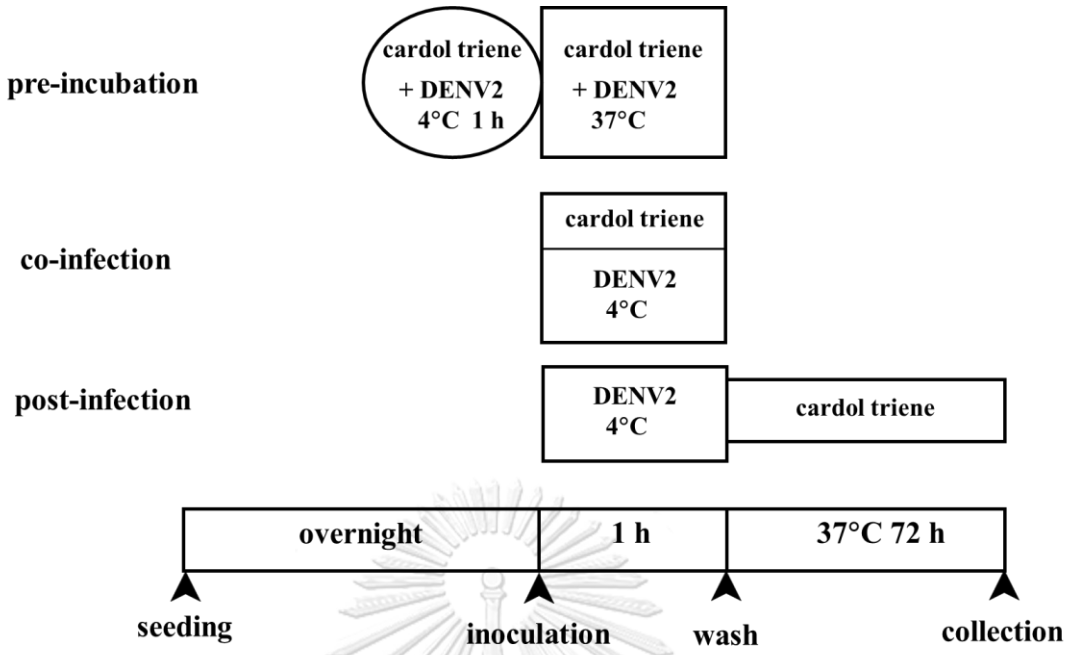


Figure 5.9 Attachment inhibition procedure diagram.

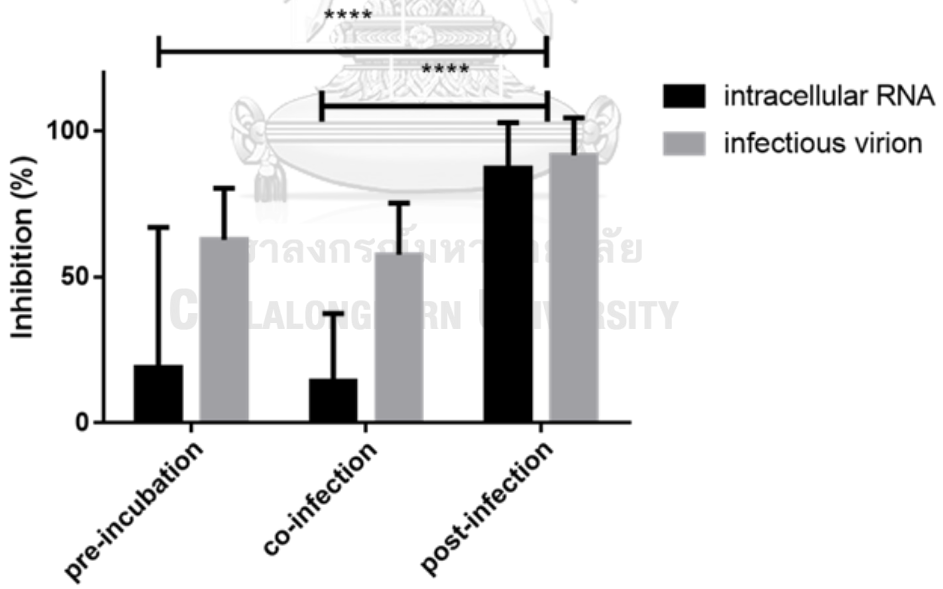


Figure 5.10 Percent inhibition of DENV2 infected Vero cells treated with 10 µM cardol triene at 48 h before quantified the amount of intracellular RNA and infectious virion. Data represent means ± standard error mean (SEM) of three independent experiment. Statistical differences are indicated as ****= p<0.0001

Based on the accumulated data, the most likely target was DENV E conformational change during pH-dependent fusion process. Fusion inhibition study (Fig. 5.11 fusion inhibition diagram) was adapted from (96),(97), and (98). Briefly, cardol triene at 10 µM was added to DENV2 (Fig. 5.12) or Zika virus (Fig. 5.13) infected C6/36 cells at the M.O.I. of 1 for 48 h before inducing acidic condition by MES. The 4G2 antibody was used as a positive inhibition control of both DENV2 and Zika virus experiments (102). Cardol triene inhibited only dengue fusion, but not Zika virus fusion, consistent with the previous findings of EC_{50s} and molecular docking. Blocking the kl-loop prevented the conformational change of DENV dimer to trimer that was required to initiate pH-dependent fusion. Therefore, we concluded that one of the targets of cardol triene, and other CNSL-derived phenolic lipids, could be the kl-loops of DENV E protein.

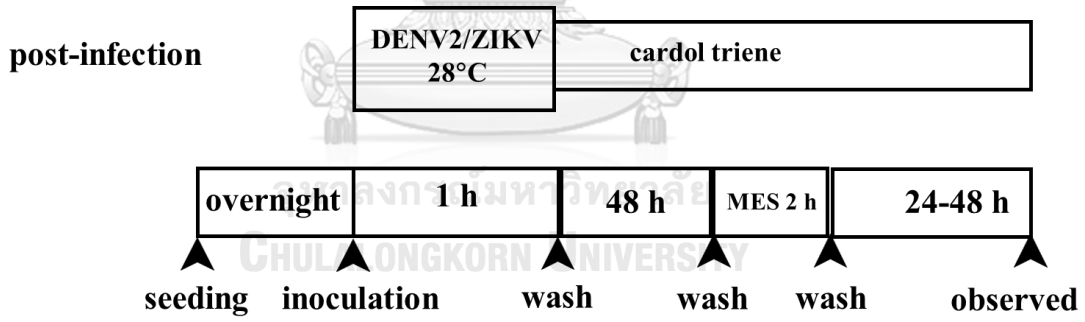


Figure 5.11 Fusion inhibition procedure diagram.

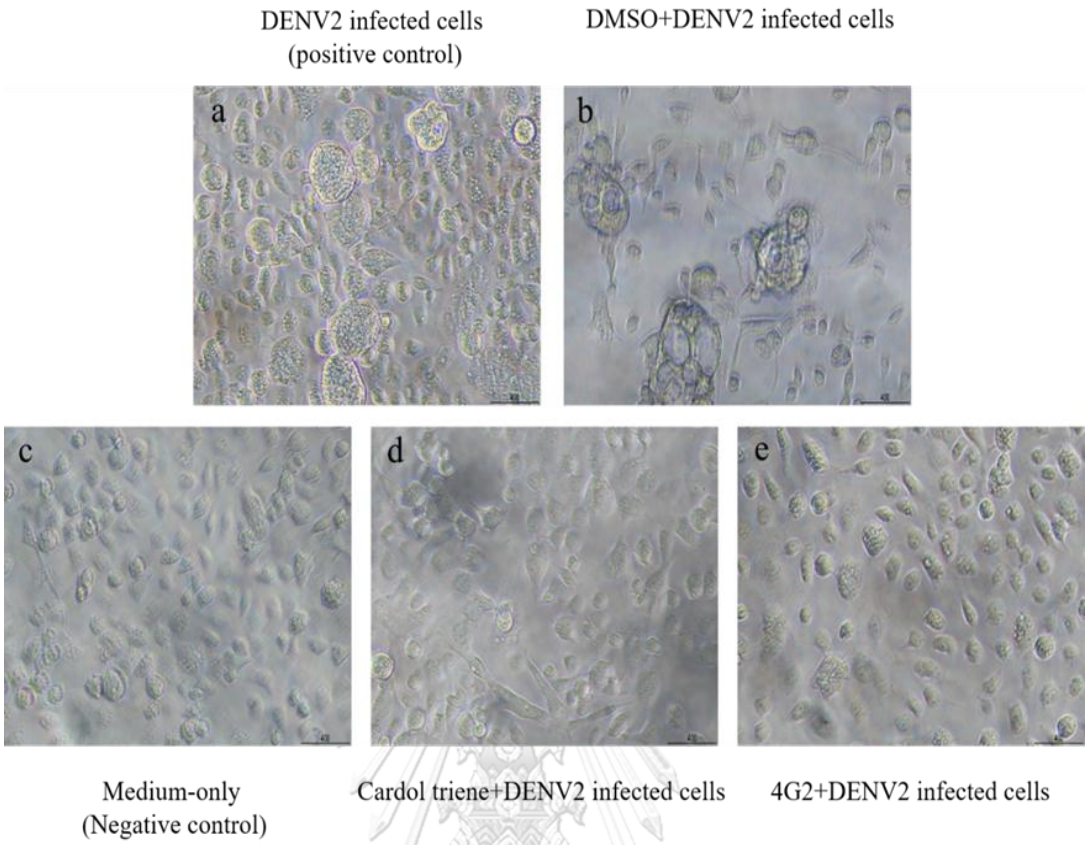


Figure 5.12 Morphology of DENV-infected C6/36 cells and treated with 10 µM cardol triene at 48h before induced fusion by MES. Images are at 400x magnification and data were representation of three independent experiment.

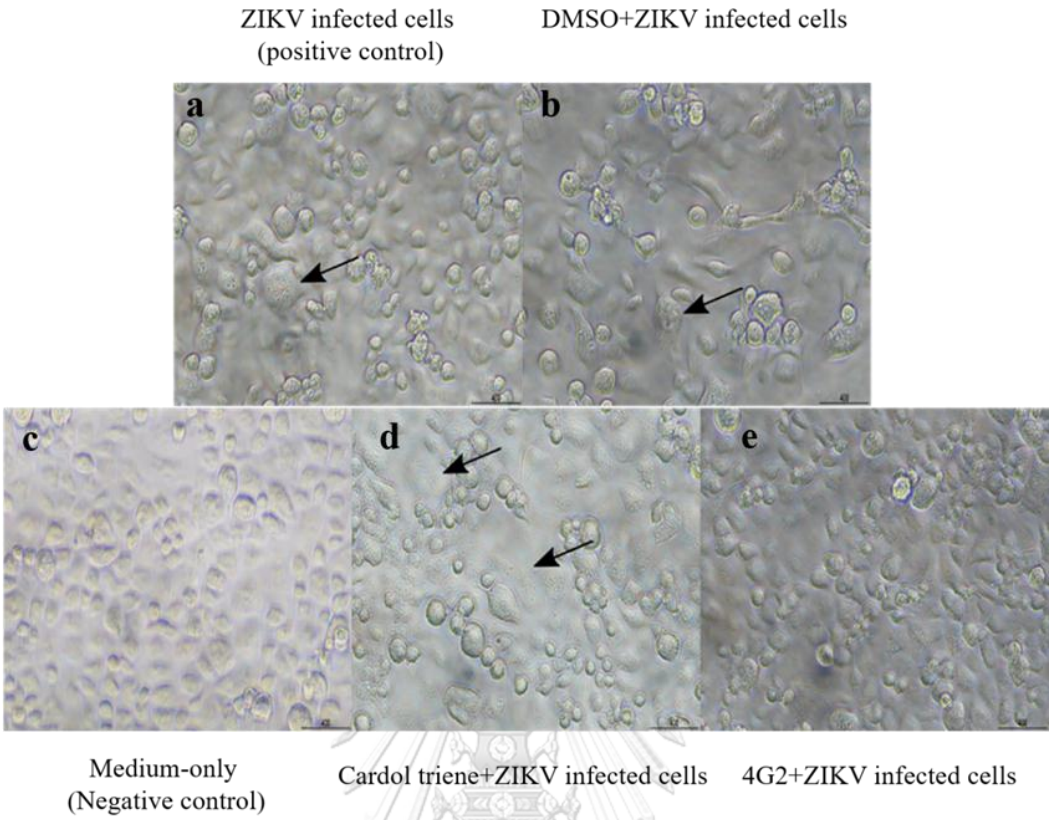


Figure 5.13 Morphology of Zika virus-infected C6/36 cells and treated with 10 µM cardol triene at 48 h before induced fusion by MES. Fused cells were indicated in a, b and d (arrows). Images are at 400x magnification and data were representation of three independent experiment.

5.4.4 To explore the possible target of the late stages:

Replicon inhibition study

We analyzed the replication inhibition using BHK-21/DENV2 replicon cells stably expressed the non-structural proteins. Cardol triene at 10 µM and 20 µM were added to the replicon cells for 72 h and the replication inhibition was accessed by RT-qPCR of DENV2 NS1. Ribavirin, a known inhibitor of flaviviral replication, was used as a positive inhibition control (103). We found 76.60 ± 7.59 percent and 89.44 ± 4.02 percent inhibition from 10 µM and 20 µM cardol triene, respectively (Fig. 5.14a), comparable to those of ribavirin. The inhibitory effect at 20 µM of cardol triene was closed to ribavirin that showed 98.42 ± 0.68 percent and 98.09 ± 0.72 percent at 10 µM and 20 µM, respectively. Replicon cell viability under cardol triene treatment was also analyzed using an MTS assay. Cardol triene treatment at 10 µM and 20 µM for 72 h did not have effect on cell viability (Fig. 5.14b). Therefore, we concluded that cardol triene inhibited DENV RNA replicon replication. Results from this study the replication could be the targets of cardol triene.

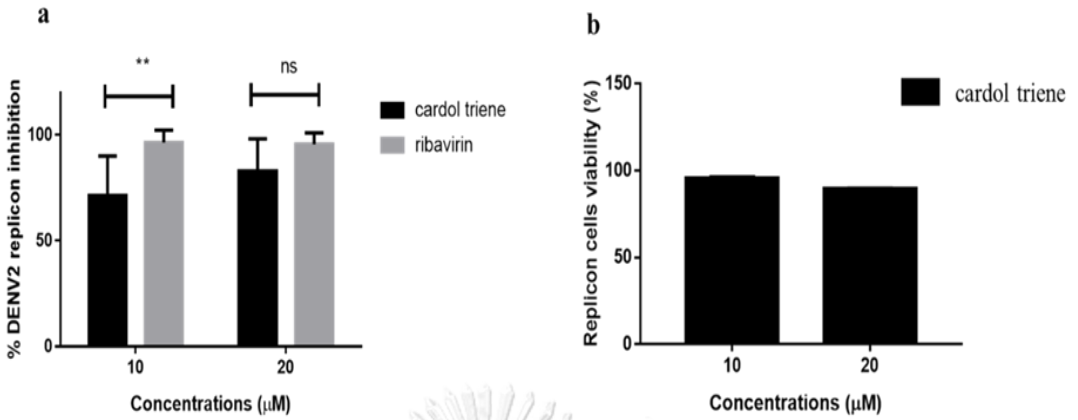


Figure 5.14 Replicon inhibition study. (a) Percent replicon cells were inhibited by cardol triene (black bars) and ribavirin (gray bars) at 72 h. (b) Percent replicon cells viability were treated with 10 and 20 μM cardol triene for 72 h. Data were represent of three independent experiment. Statistical differences are indicated as ** p-value<0.01, ns= not significant.

In conclusion, this study characterized the CNSL-derived phenolic lipids as dengue virus inhibitors. Among the 11 homologues, cardol triene showed the broadest therapeutic index so it was chosen for further investigations. Cardol triene expressed pan-dengue inhibition, but did not inhibit Zika virus. Also, the compound showed good cytotoxic profile towards three mammalian cell lines. Molecular targets of cardol triene were likely a kl-loop of DENV E protein preventing fusion at the early stages. Moreover, the screening result from the replicon inhibition study showed the replication could be the target at late stages.

CHAPTER6
DISCUSSION

Phenolic lipids, the natural amphiphilic long chains with saturated and unsaturated homologues, were first reported in *Ginkgoaceae* and *Anacardiaceae* plants with versatile biological activities (104). In this work, all the main components of phenolic lipids were isolate from cashew nut shell liquid (CNSL). The efficacies of viral inhibition were analyzed using Vero cells and DENV2 and cardol triene was the compound showing highest TI value. Cardol triene was then further investigated for efficacies against DENV1, 3, 4, and Zika virus. Results showed that the compound inhibited all dengue viruses but had no effect over Zika virus. Cytotoxicities against three human-derived cell lines were also studied and results showed that cardol triene was less cytotoxic than cardol mixture in all tested cell lines. Moreover, the mechanism of action was also investigated and results showed that the molecular targets were likely at the kl-loop of DENV envelope protein. Our findings showed for the first time that CNSL-derived phenolic lipid had a potential for further development as anti-dengue inhibitors.

All homologues of CNSL-derived phenolic lipids showed similar efficacies within against DENV2 (Table 5.2). We then hypothesized that all CNSL-derived phenolic lipids shared the same molecular targets on DENV2. Moreover, anacardic acid saturated (C15:0) that inhibited DENV2 with the EC_{50} of 4.31 ± 1.71 µM was also reported to inhibit hepatitis C virus with the EC_{50} of 7.25 µM (79). In contrast, cardol triene inhibited DENV1-4 but had no effect over Zika virus. Based on the findings, we speculated that the similar efficacies of DENV2 and HCV

might be a coincidence of the compound targeted different molecules. Evidences on the study of mannoside glycolipid conjugates suggested that the saturated and unsaturated C24 tail were actively inhibited DENV2 infection (105). Further structure-activity relationship study using MD simulation of kl-loop with a variety of phenolic lipids including another head groups, tail length (C15-C25), and number of tail double bonds should be performed.

Cardol triene showed cytoprotective effect towards Vero, HepG2, THP-1, and HEK-293 cell lines with the CC_{50} of 207.30 ± 5.24, 140.27 ± 8.44, 129.77 ± 12.08, 92.80 ± 3.93 µM, respectively (Figure 5.5) (Saelee, T, unpublished). The cytoprotective effect of cardol triene was also reported in normal human lung fibroblast cell line, GM07492A, with the 192.6 ± 6.0 µM (106). Cardol monoene, however, was reported with a cytotoxicity against the SW620 (IC_{50} of 14.18 ± 0.76 µM), KATO-III (IC_{50} of 19.06 ± 0.39 µM), Hep-G2 (IC_{50} of 2.23 ± 0.22 µM), Chago I (IC_{50} of 2.55 ± 0.18 µM) and BT474 (IC_{50} of 13.46 ± 0.14 µM) cell lines (80, 86). By similar to the anacardic acid saturated was reported with a cytotoxicity against the K562 (IC_{50} of 25.40 µM) and AGS (41.60 µM) cell lines (107). Therefore it was suggested as anti-cancer chemotherapy. Moreover, we noticed that the increasing tail double bonds of anacardic acids, cardanols, and cardols correlated with higher CC_{50} value (Table 5.2). It is possible that the increasing double bonds of the tails would facilitate the compound incorporation into lipid bilayer and stabilizing the bilayer by increasing the membrane fluidity (108). Moreover, among the three head groups, cardol showed the highest cytoprotection possibly because cardol had a rod-like shape with adequate hydrophobicity for insertion and stabilization into the hydrophobic parts of bilayer. Anacardic acids had a conical shape and more

hydrophilic than cardol, therefore it was incorporated into the subsurface and introduced a constraint on lipid packing leading to earlier membrane disruption (78, 88).

The major target of cardol triene, and potentially other phenolic lipids, was likely the kl-loop of DENV E protein. This target was one of the hotspots in dengue drug discovery and a variety of ligands were reported as fusion inhibitors (97, 109-111). Cardol triene was structurally similar to ß-OG, the crystalized ligand, and similar binding moieties were identified. For a clear demonstration of the compound failure to inhibit ZIKV-induced fusion, we plan to perform the blue-fluorescent DAPI nucleic acid stain. And further SAR studies would enlighten the drug design for the next generation of phenolic lipids as dengue inhibitors.

Our results showed that replicons RNA were inhibited by cardol triene. The replicon RNA was a self-replicating viral RNA but did not produce the infectious virions due to absence of structural genes (112, 113). Our results was in accordance withprevious report that anacardic acid saturated inhibited hepatitis C viral replication (79). Since this group of compounds are potential for multiple targets related to DENV replication by both host and viral factors. However based on the accumulated data from EC_{50s} and fusion study showed cardol triene have selective efficacies between DENV and Zika virus. Further studies should to explore the exact molecular target of phenolic lipid compounds with two viral proteins by *in silico* molecular docking, the chemical affinity-tagged purification and identification such as chloro-alkane tagging, azide-alkyne streptavidin-biotin system or liquid chromatography mass spectrophotometry system.

Moreover, previous reports showed that the longer and the more unsaturated hydrocarbon tail bonds related to higher affinity to either disruptive and stabilizing the lipid bilayer (78, 88) (87, 114). It is also possible that membrane insertion is one of the strategy of cardol triene to protect the cell membrane, thus tremendously increasing CC50s in all tested cell lines. In addition, a previous report suggested that the incorporation of phenolic lipids into lipid membrane could promote liposome fusion to the cell membrane and could activate the release of drug molecules from the interior of liposomes to the target cells (87). In our case, cardol triene itself was also active as anti-dengue by blocking the DENV E conformational change. Therefore, cardol triene could possibly being directly delivered to DENV-inhabitating endosome via membrane incorporation and liposome fusion, which subsequently bind to kl-loop as previously described.

Naturally, lipids are commonly the component of cellular membrane but some lipid call bioactive lipids (e.g. diacylglycerols (DAG), eicosanoids, and sphingolipids respectively) are important to regulate the cellular pathways and signaling events (108). It was possible that phenolic lipids would mimic the functions of bioactive lipids. Further studies to explore a potential antiviral activities of phenolic lipids as bioactive lipid could be performed. Moreover, it is possible that phenolic lipids could activate pattern-recognition receptors (PRRs) as foreign pathogen-associated molecular pattern (PAMPs). However, Vero cells is a cell-line that lacks interferon type 1 system (115) to respond to such pathway. It was then unlikely that the viral inhibition would be the results of activating the innate immune response.

The phenolic lipid compound are one of the main interested in pharmaceutical. The application of phenolic lipid compound is limited due to poor solubility and low bioavaibility. Utilizing liposome vehicle might be a solution of drug administration as this strategy has a potential to deliver a lipophilic drug between membranes without perturbing their membrane stability. Another possible application is encapsulation in order to reduce the side effected and prevent the drug form exposure to the digestive system (116).

In conclusion, we demonstrated for the first time that phenolic lipids including anacardic acid, cardanol, and cardol were potential candidates for drug development. The compounds inhibited all serotypes DENV with good efficacy, and mild cytotoxic effects. The molecular targets were identified at a kl-loop of E protein at the early stage. The SAR of phenolic lipids should be further explored for potentials to be antiviral agents for their versatile possible targets. Moreover, its selective efficacies towards DENV but not Zika virus could become a crucial tool for structural and functional comparative studies. Since this group of compounds are relatively new in antiviral drug discovery, a lot of knowledge is awaited to be explored and discovered. And we clearly showed in this research that this group of compounds has strong potentials to become novel antivirals.

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APPENDIX

APPENDIX A

REAGENTS, MATERAILS AND INSTRUMENTS

Reagents

Absolute methanol	(Merk, Germany)
Crystal violet	(Merk, Germany)
Dimethyl sulfoxside	(Merk, Germany)
Direct-zol^{TM} RNA MiniPrep	(Zymoresearch, USA)
EDTA	(Bio Basic Inc., Canada)
Fetal bovine serum	(GIBCO, USA)
Formaldehyde	(CARLO ERRA, Italy)
Geneticin (G418)	(Bio Basic Inc., Canada)
Gum tragacanth	(Sigma Aldrich, USA)
HEPES	(Bio Basic Inc., Canada)
Isopropanol	(Merk, Germany)
M199 medium	(GIBCO, USA)
MEM medium	(GIBCO, USA)
Methanol	(Merk, Germany)
MES (N-morpholino ethanesulfonic acid)	(Sigma Aldrich, USA)
MTS reagent	(Promaga, USA)
Na_2HCO_3	(Sigma Aldrich, USA)
Penicillin G	(Bio Basic Inc., Canada)
Potassium chloride	(Merk, Germany)
Potassium phosphate	(Bio Basic Inc., Canada)
Power SYBRGreen PCR Master Mix	(ABI7500, USA)

Ribavirin	(TargetMol, USA)
Sodium bicarbonate	(Sigma Aldrich, USA)
Sodium chloride	(EMSURE, Germany)
Sodium phosphate	(Merk, Germany)
Streptomycin	(Bio Basic Inc., Canada)
TRIZOL reagent	(Ambion, USA)
Trypsin	(Bio Basic Inc., Canada)

Materials

Centrifuge tube (15 and 50 ml)	(JET BIOFIL, China)
Microcentrifuge tube (1.5 ml)	(JET BIOFIL, China)
Flat 24, 96 well-plate	(SPL LIFE SCIENCE, Korea)
Tissue culture flask (T25 and T75)	(NUNC, Denmark)

Instruments

Autoclave (model-SX-700)	(Tomy, Japan)
Biophotometer (D30)	(Eppendorf, USA)
Centrifuge (Biofuge Stratos)	(SORVALLR, Germany)
CO_2 incubator	(Thermo Forma, USA)
Incubator	(Memmert, Germany)
Inverted microscope (Eclipse TS100)	(Nikon, USA)
Microcentrifuge (model: Forc 1418)	(Edison, USA)
Microplate reader (model: VICTOR^{TM}X3)	(PerkinElmer, USA)
Mixer-vortex	(Science industrial, USA)
Step-OnePlus Real-time PCR System	(Applied Biosystems, USA)
Water bath	(Julabo, Germany)

APPENDIX B

REAGENTS PREPARATION

Reagents and media for cell culture

2X MEM

MEM with L-glutamine	19.2	g
Sterilized DDW	1000	ml
Sterilized by filtration and stored at 4°C		

2X M199

M199 with L-glutamine	19	g
Sterilized DDW	1000	ml
Sterilized by filtration and stored at 4°C		

10% MEM (Growth media for LLC/MK2 cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and		
Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Sterilized DDW	36.5	ml
Stored at 4°C		

10% MEM (Growth media for BHK-DENV2 replicon cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	500	µl
Geneticin (G418) (50 mg/ml)	600	µl
10% Na_2HCO_3	2	ml
Sterilized DDW	36.5	ml

Stored at 4°C

10% MEM (Growth media for C6/36 cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	700	µl
Penicillin (100 I.U./ml) and		
Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	500	µl
Sterilized DDW	36.5	ml

Stored at 4°C

10% M199 (Growth media for Vero cells)

2X M199 with L-glutamine	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and		
Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml

Sterilized DDW	36.5	ml

Stored at 4°C

1% MEM (Maintenance medium for LLC/MK2 cells and BHK-DENV2 replicon cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	1	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Sterilized DDW	45.5	ml

Stored at 4°C

1% MEM (Maintenance medium for C6/36 cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	1	ml
10 mM HEPES	700	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	500	µl
Sterilized DDW	45.5	ml

Stored at 4°C

1% M199 (Maintenance medium for Vero cells)

2X M199 with L-glutamine	50	ml
Fetal bovine serum	1	ml
10 mM HEPES	500	µl

Penicillin (100 I.U./ml) and

Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Sterilized DDW	45.5	ml

Stored at 4°C

5% Trypsin

Trypsin	5	g
Sterilized DDW	100	ml

Sterilized by filtration and stored at 4°C

0.25% Trypsin-EDTA

5% Trypsin	2	ml
1% EDTA	800	µl
1X PBS	37.2	ml

Stored at -20°C

0.05% Trypsin-EDTA

0.25% Trypsin	4	ml
1% EDTA	320	µl
1X PBS	15.68	ml

Stored at 4°C

1% EDTA

EDTA	1	g
Sterilized DDW	100	ml

Sterilized by filtration and stored at 4°C

10X PBS pH 7.4

NaCl	40	g
KCl	1	g
Na_2HPO_4	5.75	g
KH_2HPO_4	1	g
Sterilized DDW	500	µl

Sterilized by autoclaved and stored at room temperature

1X PBS

10X PBS	20	ml
Sterilized DDW	180	ml

Stored at room temperature

10 mM HEPES

HEPES	11.915	g
Sterilized DDW	50	ml

Sterilized by autoclaved and stored at 4°C

10% Na_2HCO_3

Na_2HCO_3	5	g
Sterilized DDW	50	ml

Sterilized by autoclaved and stored at 4°C

0.5 M MES (N-morpholino ethanesulfonic acid)

MES	0.98	g
Sterilized DDW	50	ml

Sterilized by filtration and stored at room temperature

Plaque overlay medium

1.6% Gum tragacanth

Gum tragacanth	1.6	g
Sterilized DDW	100	ml

Sterilized by autoclaved and stored at 4°C

0.8% Overlay medium

2X MEM with L-glutamine	50	ml
1.6% Gum tragacanth	50	ml
Fetal bovine serum	1	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and		
Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	4	ml

Stored at 4°C

1% Crystal violet staining dye

Crystal violet	1	g
5% Isopropanol	5	ml
10% Formaldehyde	25	ml
Sterilized DDW	70	ml

Stored at room temperature

APPENDIX C

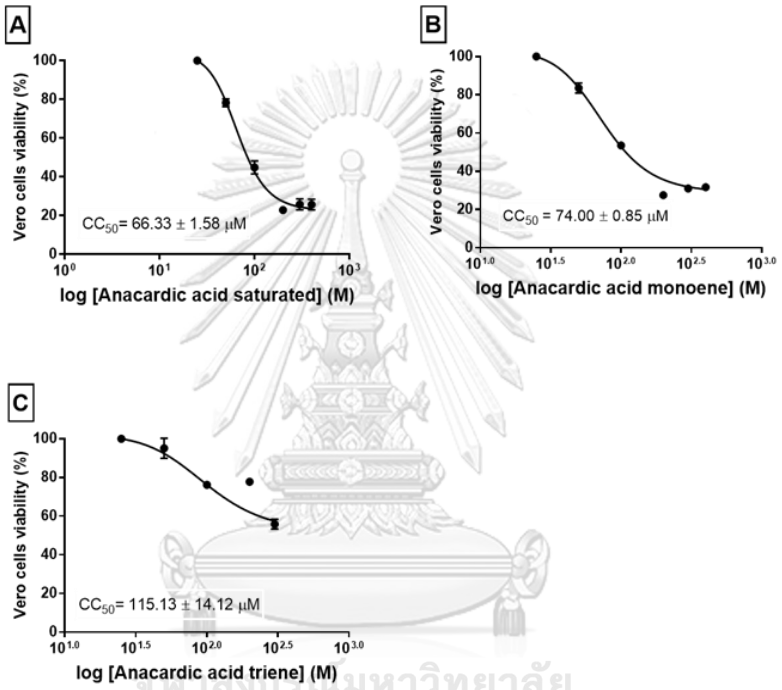
PRIMERS SEQUENCES

Primers	Sequences
DN-F	5’-CAA TAT GCT GAA ACG CGA GAG AAA-3’
DN-R	5’-CCC CAT CTA TTC AGA ATC CCT GCT-3’
D2-F NS1	5’-CTG CGA CTC AAA ACT CAT GTC AG-3’
D2-R NS1	5’-GGC TTT CTC TAT CTT CCA TGT GTC-3’

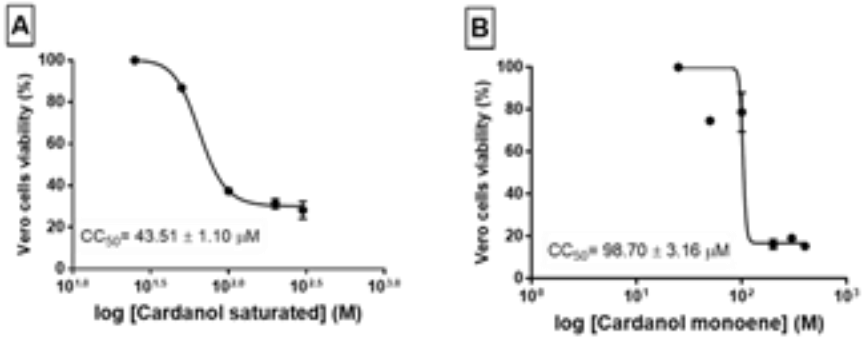
APPENDIX D

CYTOTOXIC CONCENTRATION CC_{50} OF PHENOLIC LIPD COMPOUNDS

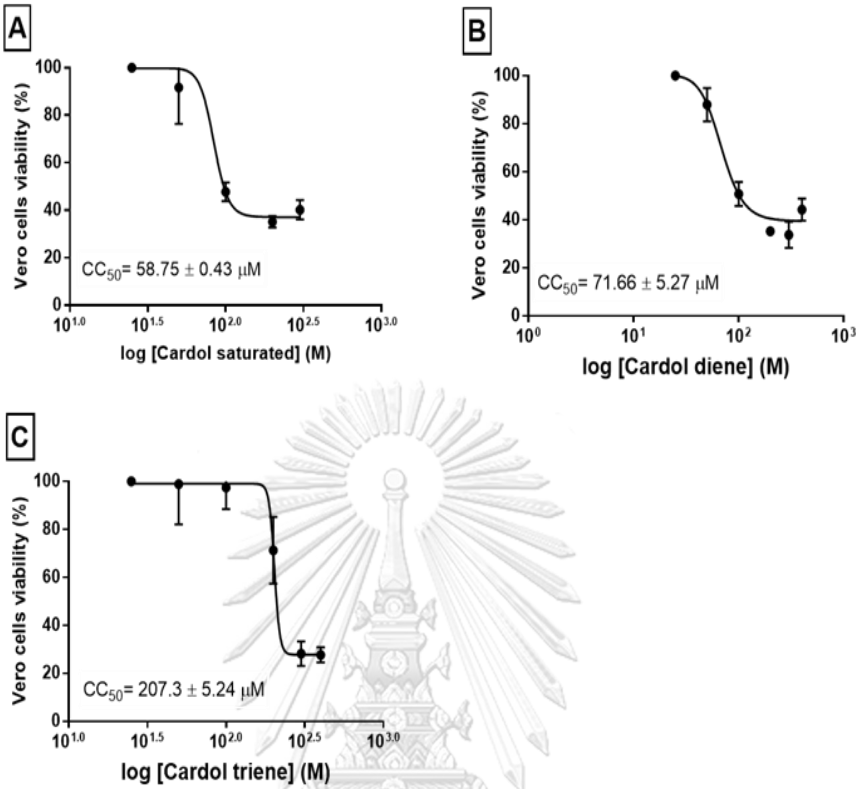
Cytotoxic concentration (CC_{50}) of Anacardic acid



Cytotoxic concentration (CC_{50}) of Cardanol

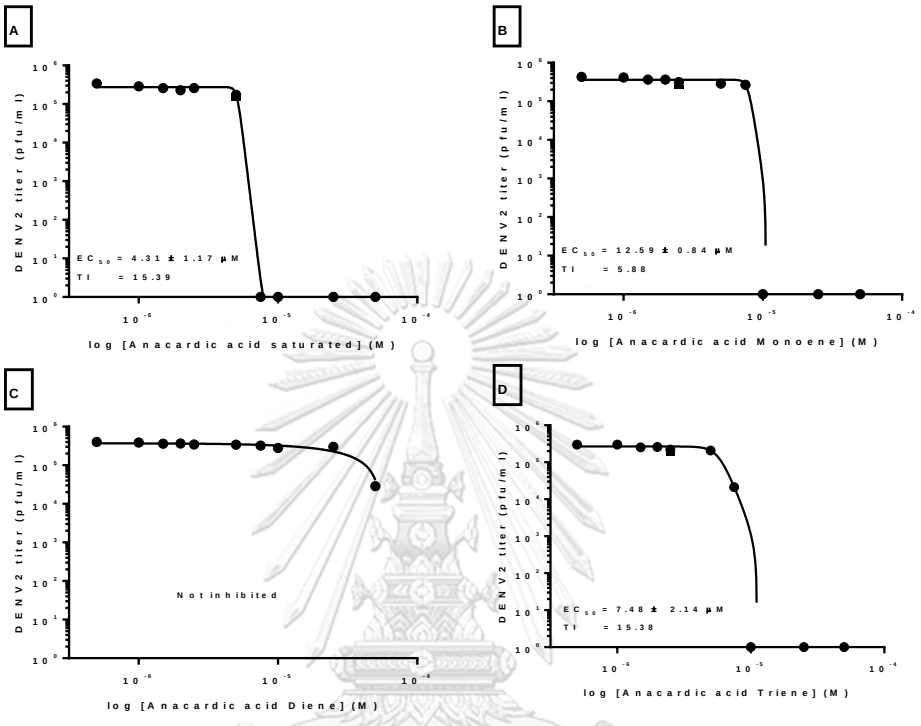


Cytotoxic concentration (CC_{50}) of Cardol

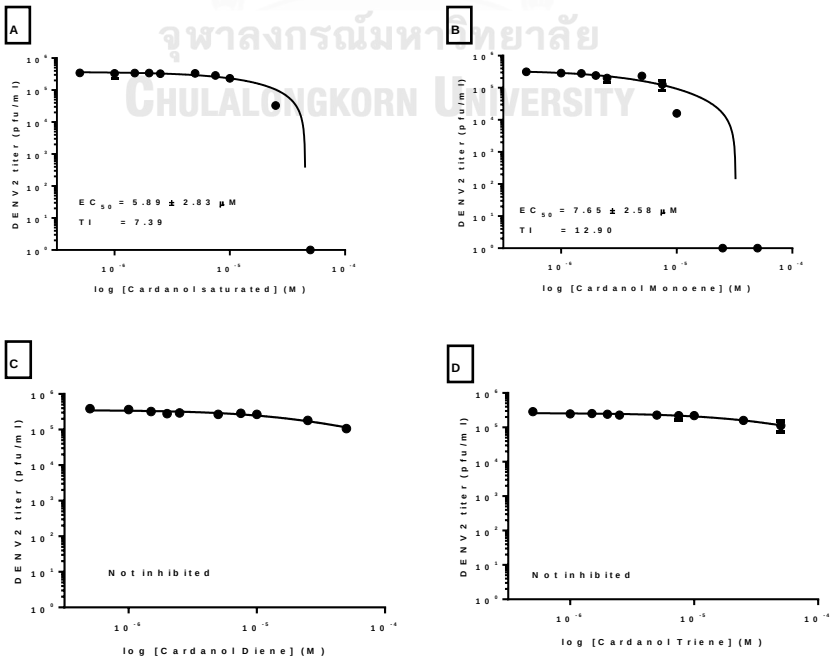


EFFECTIVE CONCENTRATION (EC_{50}) OF PHENOLIC LIPID COMOUNDS

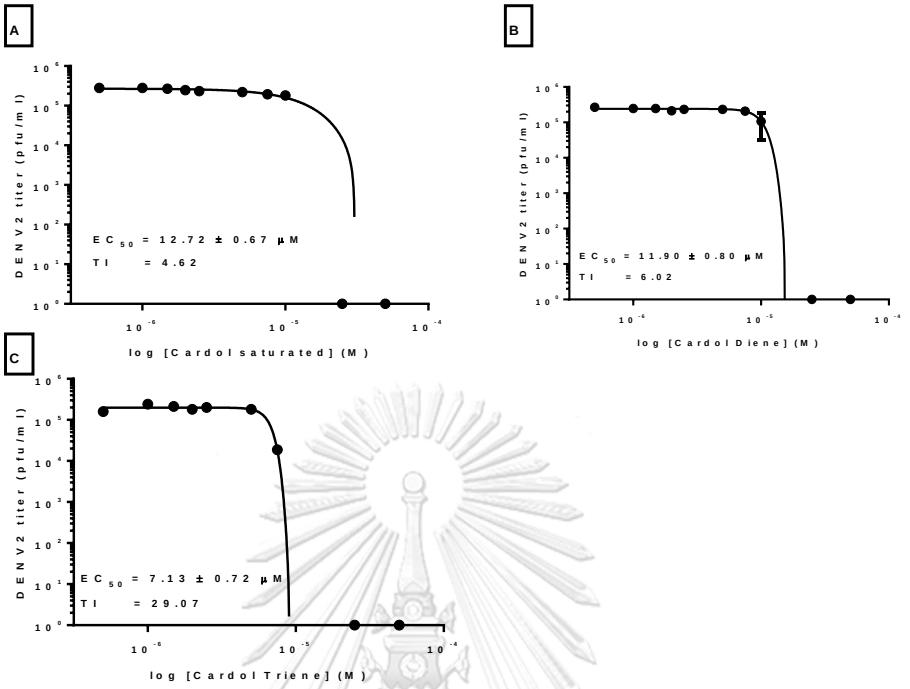
Effective concentration (EC_{50}) of anacardic acid



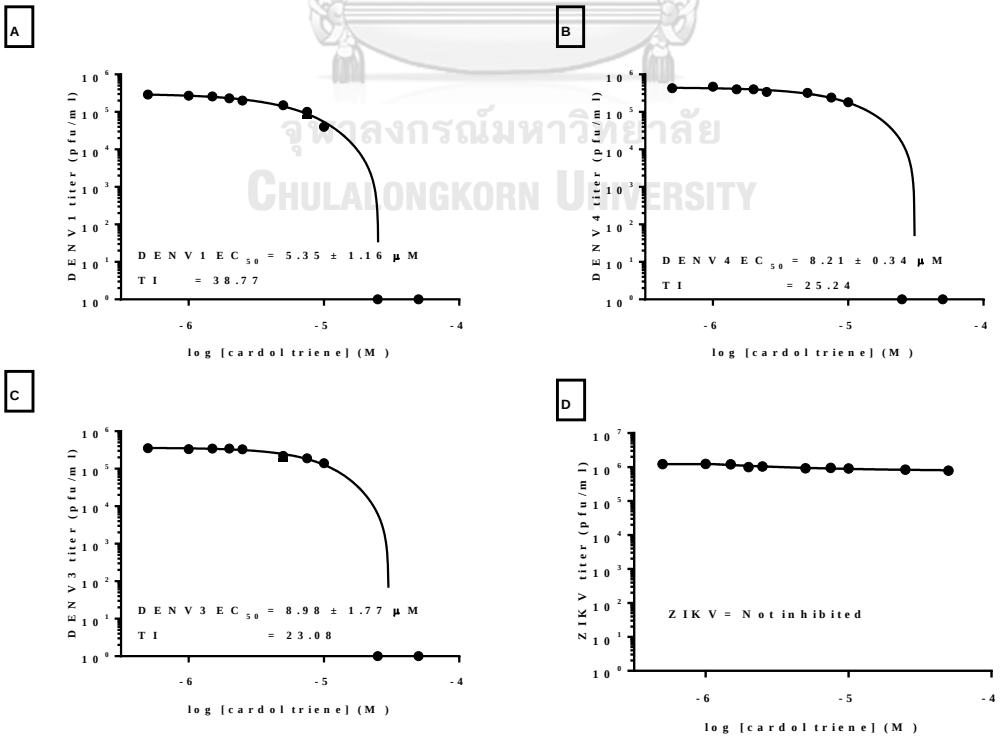
Effective concentration (EC_{50}) of cardanol



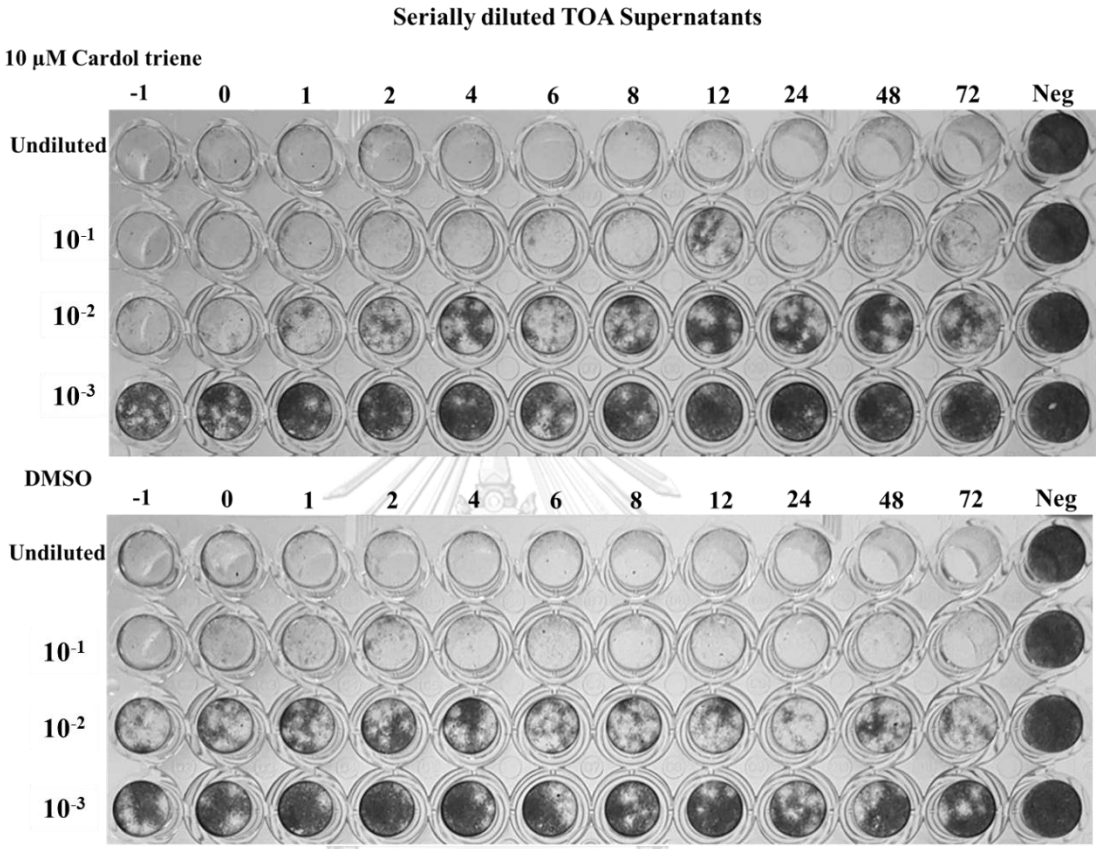
Effective concentration (EC_{50}) of Cardol



Effective concentration (EC_{50}) of cardol triene with DENV 1, 3, 4 and Zika virus

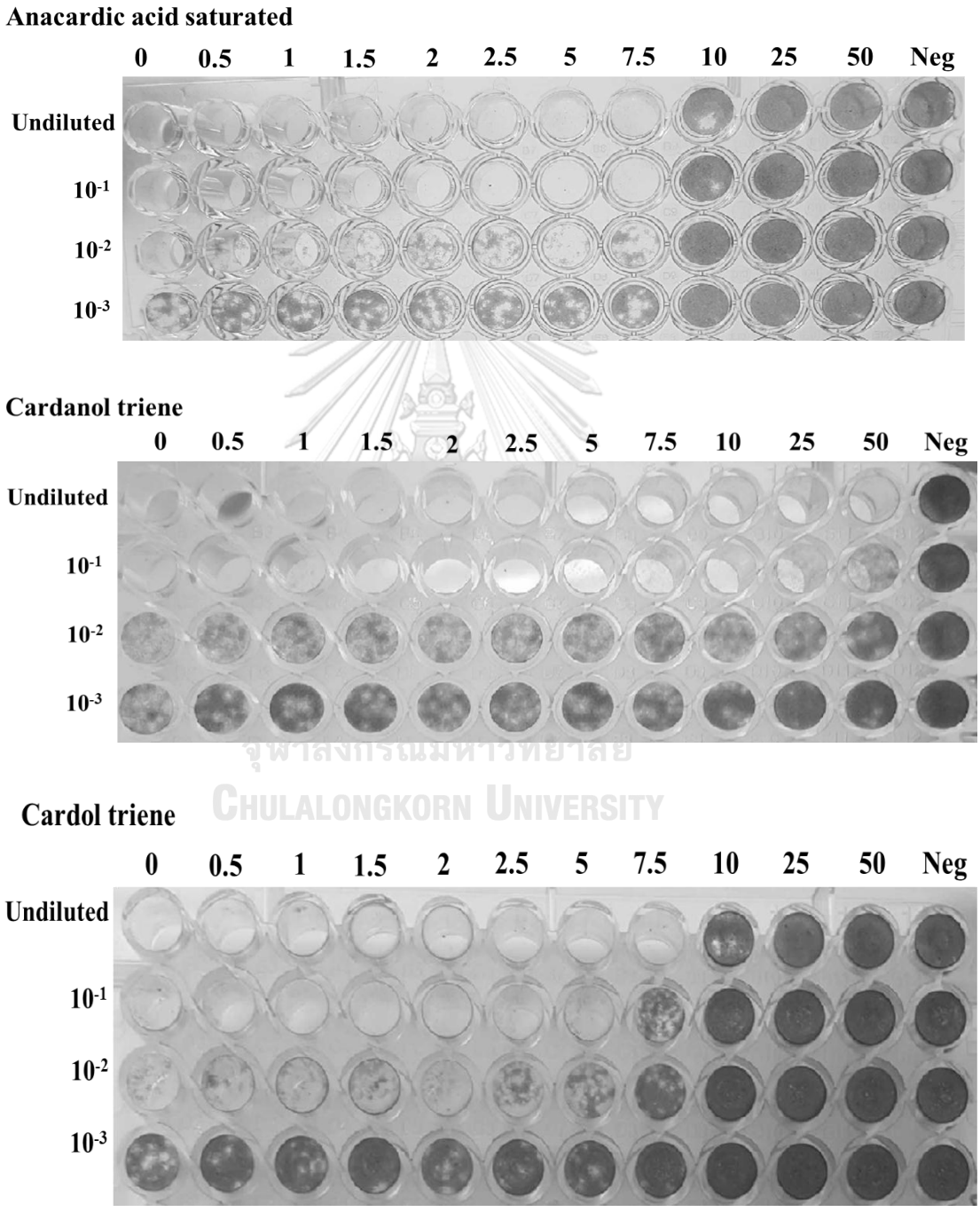


PLAQUE FORMATIONS FROM TOA SUPERNATANTS AFTER 10 µM CARDOL TRIENE TREATMRNT

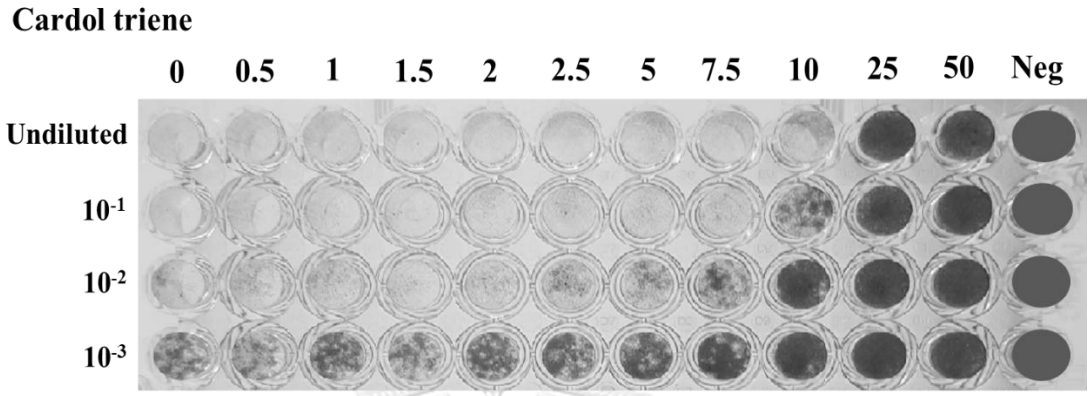


EC_{50} PLAQUE FORMATIONS OF DENV 1-4 AND ZIKA

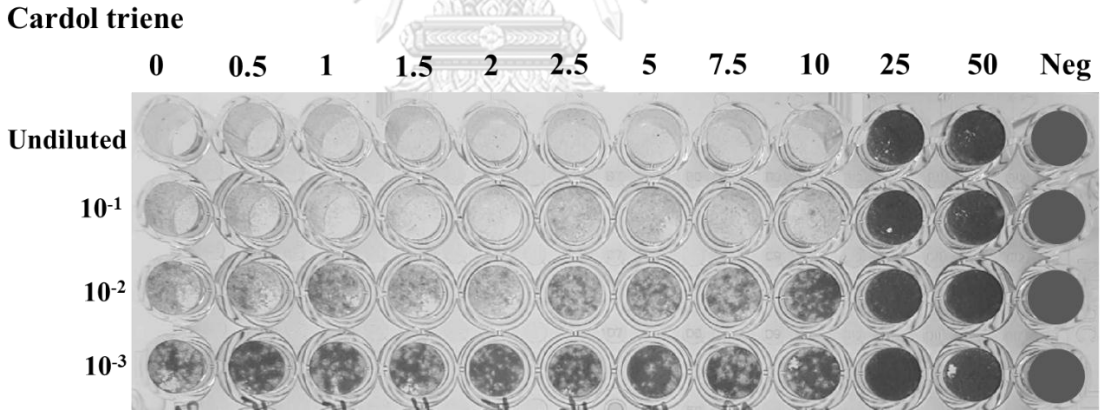
Serially diluted DENV2 supernatants in LLC/MK2 cells of representative plate



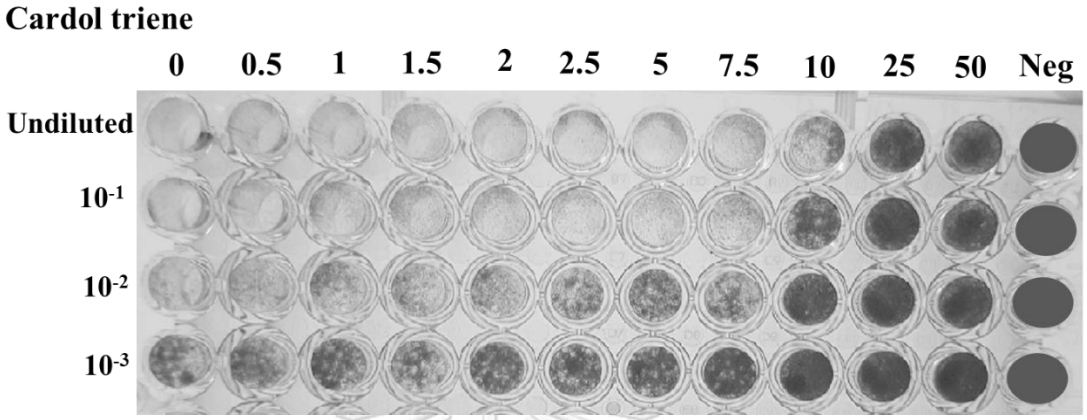
Serially diluted DENV1 supernatants in LLC/MK2 cells of representative plate



Serially diluted DENV3 supernatants in LLC/MK2 cells of representative plate



Serially diluted DENV4 supernatants in LLC/MK2 cells of representative plate



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