CHAPTER V
DISCUSSION

Despite an increase in the understanding of DV molecular biology, immunology, and pathology, little progress has been made in the area of therapeutics for DF and DHF. Currently, no effective anti-viral agent is available for the prevention or treatment of infections with DV [210]. The requirements of an anti-DV therapeutic should be nontoxic, inexpensive, easy to administer, stable for months at variable temperatures, and effective against all four DV serotypes.

A great of medicinal plants have been used to treat viral infections for a long time because of their properties including chemical diversity, structural complexity, lack of substantial toxic effects, and broad spectrum. It has been established that many of them inhibit several steps of the viral replication cycle of many DNA and/or RNA viruses. These anti-viral activities make them ideal candidates for new therapeutics [211, 212]. In addition, the multiple steps in DV replication cycle such as viral entry, viral membrane fusion, genome replication, virus particle assembly and maturation are all potential anti-viral targets.

The present work aimed to evaluate anti-DV activity of compounds prepared from Thai medicinal plants including chlorophyll a and chlorophyll b related compounds (7 compounds) which were extracted from the aerial parts of the plant (leaves and stems) of *C. nutans (Burm. f.) Lindau (Family Acanthaceae)* [207] and are well-known medicinal plants widely used in Thai traditional medicine as anti-hepatitis and anti-herpes agent and as remedies for HSV and VZV lesions, anti-inflammatory agents for the treatment of insect bites and allergic responses [207, 213]. Three compounds, i.e. Andrographolide, 14-deoxy-11,12-didehydroandrographolide and 3,19- isopropylidene andrographolide, from *A. paniculata Nees, Acanthaceae* which possess a variety of pharmacological properties, including anti-pyretic, anti-inflammatory, anti-viral, anti-cancer, anti-Epstein–Barr virus (EBV) and immunostimulant activities. Andrographolide has also been shown to possess anti-

inflammatory activity by inhibiting the expression of several pro-inflammatory proteins that exhibit a NF-κB binding site in their genes [208, 214, 215].

The screening anti-DV activity by *C. nutans* (7 compounds) *and A. paniculata* (3 compounds) was evaluated in A549, a human airway type II alveolar epithelial carcinoma cell line. Anti-DV activity of these compounds was compared with a commercially available drug, ribavirin. It was observed that two from *C. nutans* (136B and 11(3)) and one from *A. paniculata* (SS2) maintained the cell viability of DV-infected cells and were proved their anti-DV activities. The results demonstrated that SS2 and 11(3) had anti-DV activities in the pre-entry step of DV replication whereas 136B inhibited DV RNA replication when this compound was added after entry step.

In this study, we investigated anti-DV activity at pre-entry step of compound SS2 and 11(3). The result of virucidal protocol (the viruses were mixed with each compound for 1 h before adsorption) demonstrated inhibitory effect by both compounds. The treatment of the cells before infection demonstrated low activity, suggesting insignificant effect of the both compounds on DV receptors of the host cells. In contrast, complete inhibition was demonstrated in adsorption step which each compound and DV were mixed and adsorbed at the moment of the infection and before the infection. No inhibitory effect was detected when compound was added at 2 h post-infection or in the internalizing step. Therefore, it is suggested that SS2 and 11(3) had virucidal activity that inhibited DV replication or mostly interfere on virus binding to the cell receptors. This result indicated one mechanism of 11(3), a chlorophyll related compounds ($C_{55}H_{70}N_4O_7Mg$) was to inhibit DV infection and corresponded to studies on the anti-viral activity of chlorophyll natural products, Utsunomiya et al. suggested that there might be differences in the mechanisms of action of these products for enveloped and nonenveloped viruses [216]. These chlorophyll derivatives (phaeophytins) were also suggested to have the inhibitory activity against HSV-1 before viral entry to the host cells. This effect might be virucidal or interference with viral adsorption or penetration [207].

A synthetic derivative of chlorophyll (CHL) in which the phytyl and methyl groups are substituted by sodium or potassium, as Chlorophyllin (CHLN) was suggested to be a potent anti-mutagenic compound against a variety of mutagens in

vitro and in vivo [217-219]. It was also shown to exhibit anti-carcinogenic activity in animal models [220-222]. For antiviral activity of CHLN, it was shown to inhibit haemagglutination of influenza (FLU), mumps and New castle disease virus and was virucidal for FLU, vaccinia virus and Sindbis virus [223-225]. More recently, it was demonstrated that CHLN protected HEp-2 cells from nuclear fragmentation induced by poliovirus (PV) [226]. For bovine HSV (BoHV-1), CHLN demonstrated efficient inhibition of replication mostly interfering on virus binding to the cell receptors [227].

This is the first report of SS2, a naturally occurring compounds of andrographolide as 14-deoxy-11,12-didehydroandrographolide, on anti-DV activity. The inhibition pattern in DV2 multiplication cycle was in pre-entry step at adsorption process, similar to 11(3), chlorophyll derivatives (phaeophytins). In previous study of our group, three analogues of andrographolide such as 14-acetyl-3,19-isopropylidene-andrographolide, 14-acetylandro- grapholide, and 3,14,19-triacetylandrographolide, significantly exhibited preinfection step activity against the HSV-1. This study confirmed that the three hydroxyl moieties play a role in the anti-HSV-1 activity of andrographolide and concluded that 14-acetyl analogues are good for blocking the viral entry [208].

The detailed functional interactions that occur during DV replication are largely unknown. DV proteins, a general understanding is emerging, regulate the virus replication cycle. These viral proteins are crucial to the various stages of entry, replication, assembly, and egress and serve as potential targets for the development of novel anti-viral drugs. Numerous studies have investigated approaches to disrupt virus infection by interfering with viral entry. Key proteins in these interactions are the E, prM, and C structural proteins. Potential entry-associated inhibitors that have been evaluated include small molecules [17, 189, 228] and peptide inhibitors [229].

NITD448, a novel structure of the fusion inhibitor, was proved to have antiviral properties during the viral entry phase of infection. GLIDE docking suggests that the carboxylation on the chromenone ring of NITD448 interacts with Lys128 and Gln52 of the E protein, with the trifluoro-phenyl motif of the molecule well-buried into the hydrophobic (beta OG pocket) [193, 230], similar to the chloro-phenyl-thiophene tail that was previously reported as dengue entry inhibitors [193]. Furthermore sulfated polysaccharide from a marine alga have effect anti-DV activity, here they found this

compound potently inhibits DV2 infection by DV2 particles bound exclusively to fucoidan, indicating that fucoidan interacts directly with envelope glycoprotein (EGP) on DV2 [205].

Therefore, compound complexation with virion or receptors on host cells seems to be one of the mechanisms of action; nevertheless, interference on virus replication cannot be ruled out. This showed, therefore, that 11(3) and SS2 is a promising compound with such versatile effects; however, much has to be more investigated on even the scope of virus and compound interaction. Improved inhibitors could be designed by substituting specific groups in the identified compound, as well as by targeting other sites such as ''site 2'' identified in this study, and this is the basis of ongoing investigations. Diamond MS et al. describe in detail the antiviral properties of mycophenolic acid (MPA), a drug currently used as an immunosuppressive agent against DV infection of human cells, using as a comparison ribavirin (RBV), a competitive inhibitor of IMP dehydrogenase that was shown previously to have a modest antiviral effect against DV *in vitro* MPA did not block the initial phase of viral translation but did interfere with viral protein synthesis in the amplification phase. At concentrations well below its use as an immunosuppressive agent, MPA potently inhibited DV replication and protein production. An analysis of the mechanism suggested that MPA inhibited DV infection by preventing replication of both positive-and negative-strand viral RNA [231]. The inhibitory effects of MPA is not a nucleoside analog and thus cannot incorporate into the nascent RNA strand, are reversed by guanosine, and deplete intracellular guanosine levels [232].

In our study, compound, 136B, a chlorophyll derivatives ($C_{55}H_{74}N_4O_5$) showed the inhibitory effect of DV viral RNA replication when this compound was added after infection. After adsorption and internalization, DV multiplication cycle consists of many steps and the detailed functional interaction is unknown. The nonstructural (NS) enzymes of the DV replication complex include the NS3 protease and with its NS2B cofactor, the NS3 helicase/nucleoside triphosphatase (NTPase)/RNA 5' triphosphatase (RTPase), and the NS5 methyltransferase/RNA-dependent RNA polymerase. These proteins serve as potential inhibitory targets for antiviral agents since they are required for virus replication. Supporting this information, in 2006, Siewkiat T and Pippen R [166] performed bioassay for screening of DV2 NS2B/NS3

protease inhibition of the crude extracts and the methanol and hexane partitioned fractions from the rhizomes of six Zingiberaceae comprising five *Curcumas* and one *Zingiber* using Fluorogenic Peptides. They found the methanol fractions of *Curcuma longa* (L.), *Zingiber zerumbet* exhibited inhibiting effect of DV2 NS2B/NS3 protease. For compound 136B, identification of target site should further investigated.

The extracts from C. *nutans (Burm. f.) Lindau (*Family *Acanthaceae*) and *A. paniculata* have variety of pharmacological properties, some immune-modulated properties of chlorophyll related compounds from *C. nutans (Burm. f.) Lindau (*Family *Acanthaceae*) [207] that are well-known medicinal plants widely used in Thai traditional are anti-inflammatory agents for the treatment of insect bites and allergic responses [207, 213]. In contrast to properties of *A. paniculata* that has scientifically exhibits both of anti-inflammatory and immunostimulatory activity by increased proliferate ion of lymphocytes and production of interleukin 2, enhanced the tumor necrosis factor α production. Intensive studies was performed in the extracted from *A. paniculata.* By bioactivity-guided chromatographic fractionation, 23% of the AP EtOAc extract, with major compounds such as andrographolide (12%), 14-deoxy-11,12-didehydroandrographolide (6.8%) and ergosterol peroxide (3.2%) that exerting significant inhibition of NF-kB trans-activation. In this study, AP EtOAc extract was confirmed not only to inhibit NF-kB activation dose-dependently, but also TNF-a, IL-6, MIP-2 and NO production in LPS/IFN-g stimulated Raw264.7 macrophages [233].

Chao WW, et al. found that ethyl acetate fractions of *A. paniculata* (Burm. f.) Nees (Acanthaceae) (AP), Angelica sinensis (Oliv.) Diels (Apiaceae) (AS), and Morus alba L. (Moraceae) (MA) significantly decreased NF-κB luciferase activity and also the secretion of NO and PGE_2 in LPS/IFN-γ stimulated mouse peritoneal macrophages ($p<0.05$). In contrast, they did not affect IFN-γ luciferase activity or IFN-γ production in concanavalin A (Con A)-activated mouse splenocytes. This results indicated that the anti-inflammatory properties of these plant extracts might be resulted from the inhibition of pro-inflammatory mediators (e.g., NO and PGE_2, at least in part via suppression of a signaling pathway such as $NF\text{-}_kB$. They suggested that three potent bioactive TCMH species exerted significant NF-κB inhibitory activity and acted in a cell type dependent fashion [234].

Andrographolide, recognized as the most medicinally active phytochemical in AP, has been reported to inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production through suppression of inducible nitric oxide synthase (iNOS), NF-$_k$B activation by blocking the binding of NF-$_k$B oligonucleotide to nuclear proteins [235, 236]. The EtOAc extract from AP was studied to evaluate the anti-inflammatory effect in vitro NF-kB-dependent reporter activity and in vivo LPS-induced acute inflammatory murine model. This study suggests that AP can inhibit the production of inflammatory mediators and alleviate acute hazards at its optimal dosages [237]. Corresponding with our study demonstrated that at subtoxic concentration of all 7 compounds from *C. nutans* stimulated the productiontion of PGE_2 as well as all 3 compounds (SS1, SS2 and SS3) from *A. paniculata* (Figure 30). Excepting in 136B treated cells, PGE_2 level was present at low level. This result may effect from cell type or compound concentration used. Production of PGE_2 was also detected in A549 cells that may have developed a certain dependence on the up-regulated NF-κB pathway for sustained survival and may effect to stimulate DV replication [238]. Overwxpresses COX-2 and PGE_2 production were also reported in DV infected A549 cells [9]. Activated PGE_2 may protect the cell from invasion but also be used by some viruses to favor viral production.

The studies of Triptolide and tetrandrine, compounds derived from two commonly used Chinese herbs, both demonstrated anti-inflammatory and immunosuppressive effects partly through modulation of COX-2 expression and, hence, may have antiviral effects. This study found that DV infection enhanced COX-2 expression and PGE_2 production in A549 cells, similarly to the response in dendritic cells, but not in HepG2 cells. In dengue virus-infected A549 cells, NF-κB and AP-1 were also activated, and both were dose-dependently inhibited by triptolide (0.5–4 ng/ml). Tetrandrine (1–10 μM) had no similar immunosuppressive effects and, moreover, at higher concentrations, enhanced NF-κB and AP-1 activity, COX-2 expression and PGE_2 production. However, unexpectedly, tetrandrine, but not triptolide, dose-dependently suppressed DV production in A549 cells, independent of PGE_2 level. Our findings imply that triptolide and tetrandrine may tenuate DV infection in human lung cells, but through distinct pathways [9]. Corresponding with our study, compound 136B, when treated in DV infected A549 cells, inhibited not

only viral replication, but also demonstrated the immunosuppressive effect by decreasing of PGE_2 production that may be effected partly through modulation of COX-2 gene expression (Figure 32). This result suggested that inhibitory effect of 136B compound on DV infection may partly on suppression of PGE_2 production or interaction with viral protein that are necessary to viral RNA replication.

Uncaria tomentosa, a large woody vine native to the Amazon and Central American rainforests, has been medicinally used by indigenous peoples since ancient times and has scientifically proven immunomodulating, anti-inflammatory, cytotoxic and anti-oxidant activities. The anti-viral activity of *U. tomentosa* was determined viral antigen (DV-Ag) detection in monocytes by flow cytometry. The results demonstrated an inhibitory activity of DV infection [204]. Jun-Ting Liou et al. report *Triptolide* and *tetrandrine*, compounds derived from two commonly used Chinese herbs, they studied anti-viral activity by RT-PCR and immunomodulation by EMSA, both demonstrate anti-inflammatory and immunosuppressive effects partly through modulation of COX-2 expression and, hence, may have antiviral [9].

In conclusion, this results demonstrated that chlorophyll relative compound (11(3)) from C. *nutans* and 14-deoxy-11,12-didehydroandrographolide (SS2) exhibited immunostimulating property in A549 cells at subcytotoxic level and decreased viral load by inhibiting DV adsorption in pre-entry step of replication, whereas chlorophyll relative compound (136B) from C. *nutans* suppressed PGE_2 production as well as DV replication in the tested cells. This finding may be the starting point for further study in more detail of virus-compound interaction to understand the inhibitory effect of these compounds on DV infection.