CHAPTER III
RESEARCH METHODOLOGY

Materials and methods

1. Preparation of *C. nutans* and *A.paniculata* compound solution

The preparation of *C. nutans* and *A. paniculata* compounds has been described previously [207, 208]. All 7 compounds and 3 compounds were prepared from *C. nutans* and *A.paniculata*, respectively. Compounds of *C. nutans* and *A. paniculata* were dissolved in dimethyl sulfoxide (DMSO) (Amresco®; Solon, Ohio, USA) as stock solutions (2 mg/ml) that were stored at −20 °C. The stock solutions were freshly diluted to the indicated concentrations with culture medium before use in experiments. Detail of chemicals and equipments are in appendices.

2. Cell lines

C6/36, a mosquito cell line from *A. albopictus*, was cultured in Leibovitz's L-15 medium (Gibco®; Invitrogen Co, Grand Island, New York, U.S.A.) medium supplemented with 1% tryptose phosphate broth, and 10% fetal bovine serum (FBS) (Gibco®; Invitrogen™, Carlsbad, CA), and grown at 28 °C without CO_2

LLC-MK$_2$, monkey kidney cells, were maintained in Opti-Eagle's minimal essential medium (Opti-MEM) (Gibco®; Invitrogen Co, Grand Island, New York, U.S.A.) supplemented with 2% FBS with antibiotic 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/mL gentamicin, and 2.5 µg/ml fungizone, and incubated at 37°C with 5% CO_2.

A549 cells, type II human lung alveolar epithelial cell carcinoma, were maintained in Roswell Park Memorial Institute medium 1640 (RPMI-1640) (Gibco®; Invitrogen Co, Grand Island, New York, U.S.A.) with 10% FBS and antibiotic, and incubated at 37°C with 5% CO_2.

3. Virus preparation and titer determination [34, 209]

The stock of DV2-16681 strain was prepared in C6/36 cells and titrated in LLC-MK_2 cells by immunofluorescence (IF) technique.

The DV2 was propagated in C6/36 mosquito cells in Leibovitz's L-15 medium containing 10% heat-inactivated FBS and incubation was maintained at 28°C in an incubator without 5% CO_2 atmosphere for 4–7 days. The supernatants were collected, from which virus titers were determined, then stored at −80°C until use.

This technique was used to determine virus titers. The serial dilutions of viral culture supernatants of virus were placed into 24-well plates (Costar®; Corning Inc., Cambridge, New York, U.S.A.) seeded with 80% confluent LLC-MK_2 cells and incubated at 37°C for 1 h. After adsorption, cells were washed by phosphate buffered saline (PBS) solution twice and overlaid with Opti-MEM medium containing 0.8% carboxymethyl cellulose (CMC) (SIGMA®; Sigma-Aldrich, Saint Louis, Missouri, USA) and 2% FBS. After incubation at 37°C for 7 days, the DV2 infected LLC-MK_2 cells were fixed with cold acetone, 30 minutes at -20°C and stained with human serum anti-DV (1:100 dilution) followed by rabbit anti-human IgG monoclonal antibody conjugated FITC (DAKO; Glostrup, Denmark) (1:200 dilution). Immunofluorescent cells were counted under a fluorescence microscope and viral titers were calculated.

4. Cytotoxicity study of compounds from *C. nutans* and *A. paniculata* [202]

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method. This method was performed as followed;

- Seeded A549 cells with 60,000 cells/mL (100 µL per well) in a 96-well plate (Costar®; Corning Inc., Cambridge, New York, U.S.A.) and then incubated at 37°C, 5% CO_2 overnight.
- Prepared serial two-fold dilutions of *C. nutans* and *A. peniculata* compounds in Opti-MEM medium.
- Removed the growth medium from confluent A549 cells in the 96-well plate and replaced with serial dilution of *C. nutans* and *A. paniculata* compounds in maintenance medium or maintenance medium without compounds (as cells

control) 100 µl per well with three wells for each concentration and incubated at 37°C, 5% CO_2 for 72 h.

- Added 10 µl of MTT Reagent (SIGMA®; Sigma-Aldrich, Saint Louis, Missouri, USA) to each well using a repeating pipettor to final concentration of 5 mg/mL
- Mixed gently for 10 min on an orbital shaker.
- Incubated the reagent with the cell for three to four hours at 37°C in a CO_2 incubator. After incubation, the formazan produced in the cell was appeared as dark crystals in the bottom of the well.
- Aspirated the culture medium from each well carefully to prevent disruption of the cell monolayer.
- Added 100 µl of DMSO (dimethyl sulfoxide) (Amresco®; Solon, Ohio, USA) for dissolving and measureed OD at 595 nm.
- The cell viability was evaluated by the percentage of the mean value of the optical density resulting from the cell control that set 100%. The 50% cytotoxic concentrations (CC_{50}) of compounds were calculated from the mean dose-response of three independent assays.

5. Screening of anti-DV activity [202]

Anti-DV activity was evaluated by detection of viral RNA using reverse transcription polymerase chain reaction (RT-PCR).

To screen for anti-DV activity, the test was performed as followed;

- Seeded A549 cells 60,000 cells/mL (1 mL per well) in a 24-well plate and then incubated at 37°C, 5% CO_2 overnight.
- Removed the growth medium from confluent cells in the 24-well plate.
- Incubated DV2 at 0.01 multiplicity of infection (MOI) with each compound at the subcytotoxic concentration, at 37°C for 60 min. DV2 at 0.01 MOI in medium without compound was used as virus control.
- Then infected the confluent A549 cells grown in the 24-well plates.
- After adsorption, residual inoculums were replaced by maintenance medium and each compound. 200 µM of ribavirin was used as a positive control. The infected cells were incubated for 7 days at 37°C, 5% CO_2.

- After 3 days of incubation at 37°C, supernatant was collected for RNA extraction by viral nucleic acid KIT (Geneaid; Taipei, Taiwan) and the virus replication was evaluated by RT-PCR using Superscript™ III one-step RT-PCR (Invitrogen™; Invitrogen Co., Foster, California, USA). After 5 days of incubation at 37°C, the infected cells were collected for confirmation by IF technique.
- All determinations were performed twice and each in triplicate.

Viral RNA quantification by RT-PCR

RNA was extracted by viral nucleic acid KIT (Geneaid; Taipei, Taiwan) at 3 days post-infection. All tests were conducted according to the manufacturer's protocols. Briefly; added lysis buffer to cell monolayer then transfer the mixture to the column after centrifuge (nucleic acid binding). The washing buffer was added and centrifuge again. In the final step, releasing buffer was added to the column for elution viral RNA.

The extracted RNA was determined for viral RNA of DV2 as followed;

- Superscript™ III one-step RT-PCR system with platinum® Taq DNA polymerrase (Invitrogen™; Foster, California, USA) was performed.
- Viral gene specific primers were forward -5′-TCA ATA TGC TGA AAC GCG CGA GAA ACCG-3′ and reverse -5′-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3′.
- Prepared each 25 µL reaction mixture containing 5 µL of RNA sample and 1x RT-PCR buffer, and 0.2 µM for each primer, 1.6 mM $MgSO_4$, 200 µM dNTP and SuperScript™III RT/platinum®Taq mix enzyme.
- PCR amplification were performed using the following conditions:

  For 1 cycle of cDNA synthesis: 50°C for 30 min, hot start and first denature 1 cycle.

  For DNA amplification: first step of denaturation at 94°C for 2 min and then performed 40 cycles. Each cycle consisted of 95°C for 30 sec (denature), 55°C for 1 min (anneal) and 72°C for 1 min (extend) and 1 cycle of final extension 72°C for 10 min.
- PCR product detection

5 µL of amplified products were mixed with 2 µL of the gel-loading dye buffer. The mixture was loaded into 1.5%of agarose gel in electrophoresis 0.5% TAE buffer. The electrophoresis was carried through 100 volts for 30 min. The agarose gel was stained with 0.1% ethidium bromide and destained with water, after that it was visualized on a UV-transilluminator and photographed. The 511 bp of amplified product was compared to the molecular weight marker (1 kb+ DNA marker) and positive viral control.

6. Anti-viral activity in pre-entry step of viral infection

- Seeded A549 cells with 60,000 cells/mL (1 mL per well) in a 24-well plate and then incubated at 37°C, 5% CO_2 overnight.
- Incubated DV2 at 0.01 MOI with compounds at the subcytotoxic concentration at 37°C for 1 h. DV2 in medium without compounds was used virus control. Dextran sulfate sodium salt was mixed with virus and used as a positive control.
- Removed the growth medium from confluent A549 cells in the plate.
- Adsorbed confluent A549 cells with the incubated DV2 at 37°C for 1 h.
- After 1 h adsorption at 37°C, the infected cells were washed with cold PBS to remove unadsorbed viruses.
- Added growth media and incubated at 37°C for 3 days. The amount of infectious viruses were then measured by RT-PCR and confirmed by IF technique.

7. Anti-viral activity in adsorption step [202]

- Seeded A549 cells with 60,000 cells/mL (1 mL per well) in a 24-well plate and then incubate at 37°C, 5% CO_2 overnight.
- Incubated DV2 at 0.01 MOI with compounds in the subcytotoxic concentration at 4°C for 1 h. DV2 in medium without compounds was used as virus control.
- Removed the growth medium from confluent cells in the plate.
- Adsorbed confluent A549 cells with the incubated DV2 at 4°C for 1 h.
- After 1 h adsorption at 4°C, infected cells were washed with cold PBS to remove unadsorbed viruses.
- Added growth media and incubated at 37°C for 3 days. The amount of infectious viruses was measured by RT-PCR and confirmed by IF technique.

8. Pre-binding assay [202]

- Seeded A549 cells with 60,000 cells/mL (1 mL per well) in a 24-well plate and then incubate at 37°C, 5% CO_2 overnight.
- Removed the growth medium from confluent cells in the 24-well plate.
- Added compounds in the subcytotoxic concentration to the cell monolayer, incubated at 37°C for 1 h.
- Removed the compound containing medium from confluent cells.
- Adsorbed DV2 at 0.01 MOI at 37°C for 1 h. The non-treated cells were used as virus control.
- After 1 h adsorption at 37°C, infected cells were washed with cold PBS to remove unadsorbed viruses.
- Added growth media and incubated at 37 °C for 3 days. The amount of infectious viruses was measured by RT-PCR and confirmed by IF technique.

9. Anti-viral internalization assay [202]

- Seeded A549 cells with 60,000 cells/mL (1 mL per well) in a 24-well plate and then incubated at 37°C, 5% CO_2 overnight.
- Removed the growth medium from confluent cells in the 24-well plate.
- Adsorbed the confluent A549 cells with DV2 at 0.01 MOI and incubated at 4°C, for 1 h.
- Washed the cells after 1 h adsorption with growth media and subsequently incubated at 37 °C for 1 h in maintenance media with and without the compounds.
- The cells were treated with acidic PBS, pH 3 for 30 sec to inactivate non-internalized viruses.
- Added growth media and incubated at 37 °C for 3 days. The amount of infectious viruses was measured by RT-PCR and confirmed by IF technique.

10. Anti-viral activity in post-entry step of viral infection

- Seeded A549 cells with 60,000 cells/mL (1 mL per well) in a 24-well plate and then incubated at 37°C, 5% CO_2 overnight.
- Removed the growth medium from 80% confluent cells in the plate.
- Infected with DV2 at 0.01 MOI or mock (no infection) at 37 °C for 1 h.
- After adsorption, the cells were washed with PBS.
- Overlaided the cells with 2% FBS-RPMI medium with and without each compound and incubated at 37°C for 3 days.
- The amount of infectious viruses was measured by RT-PCR and confirmed by IF technique.

11. Determination of PGE_2 production by enzyme-linked immunosorbent assay (ELISA)

To evaluate viral activity on inflammatory cytokine (PGE_2) production, the confluent cells were infected with DV at 1, 0.1 and 0.01 MOI and then incubated at 37°C for 48 h, and then supernatant was collected and used for PGE_2 determination

To study activity of compounds on PGE_2 secretion, the confluent cells were incubated with maintenance RPMI medium containing each compound at subcytotoxic concentration, at 37°C for 48 h. Then supernatant was collected and determined for PGE_2.

To investigate activity of the compounds on PGE_2 production in DV infected cells, the confluent A549 cells were infected with DV at 0.01 MOI and incubated 37°C forl h. Then replaced by maintainant RPMI medium with each compound at subcytotoxic concentration and incubated at 37°C for 48 h. The supernatant was collected and determined for PGE_2.

PGE_2 was determined using ELISA method of which principle is shown in Figure 17. A commercial ELISA kit (Biotrak™; GE Healthcare, Buckinghamshire, UK) was used and all tests were conducted according to the manufacturer's protocols as followed:

- Prepared the reagents and working standards as described in manufacturer's protocols: microplate, assay buffer, standard, PGE_2, PGE_2 antibody, PGE_2

conjugate, wash buffer, 3,3',5,5' tetramethylbenzidine (TMB)/hydrogen peroxide substrate and lysis reagent.

- Pipetted each sample into the appropriate wells.
- Pipetted diluted antibody into all wells.
- Pipetted diluted conjugate into all wells.
- Covered the plate with the lid provided.
- Incubated the plate at room temperature for 1 h on a microplate shaker.
- Aspirated and washed all wells with wash buffer.
- Bloted the plate on tissue ensuring any residual buffer was removed. The washing is essential for good performance.
- Immediately pipetted 150 µL of room temperature equilibrated enzyme substrate into all wells.
- Covered the plate with the lid provided and mixed on a microplate shaker for exactly 30 min at room temperature.
- The blue colour that developed was read at 450 nm within 30 min.

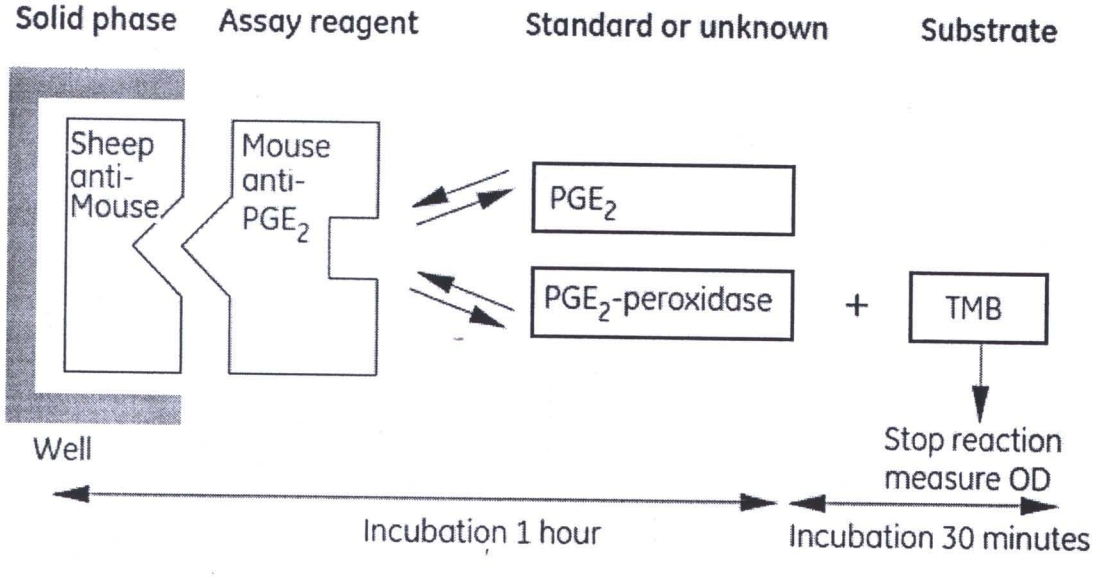

Figure 17 ELISA principle, the assay is based on competition between unlabelled PGE_2 and a fixed quantity of peroxidase-labelled PGE_2 for a limited number of binding sites on a PGE_2 specific antibody.

12. Determination of COX-2 gene expression by real-time PCR

Activity of compound on COX-2 expression was investigated in DV infected cells as followed:

Sample preparation

- Seeded A549 cells with 60,000 cells/mL (1 mL per well) in a 24-well plate and then incubated at 37°C, 5% CO_2 overnight.
- Removed the growth medium from confluent A549 cells in the 24-well plate.
- The confluent A549 cells were adsorbed with and without DV2 at 0.01 MOI, after that the infected A549 cells were incubated with compounds in subtoxic concentration.
- COX-2 gene expressions were analyzed at 24 h post-infection. The COX-2 gene expression was compared to cell control and DV-infected A549 cells without treatment.
- For analysis of COX-2 mRNA in A549 cells, all treated wells were washed with PBS one time before RNA extraction.

RNA extraction

Total RNA was prepared using TRIzol®Reagent (Invitrogen™, Carlsbad CA, USA) at 2 day post-infection. Reagents were chloroform, isopropyl alcohol, 75% ethanol and RNase-free water. All tests were conducted according to the manufacturer's protocol as followed.

- Homogenization: lysed cells directly in a culture plate by adding 800 µL of TRIZOL
- Phase separation: Added 200 µL of chloroform to homogenized samples, shaked tubes by hand for 15 sec and incubate at 25°C for 3 min.
- Centrifuged the samples at 12,000 x g for 15 min at 4°C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase.

- RNA precipitation: Transfered the aqueous phase to a fresh tube, precipitated the RNA from the aqueous phase by adding isopropyl alcohol 500 µL. Incubated samples at -70°C for 30 min and centrifuged at 12,000 x g for 10 min at 4°C.
- RNA wash: removed the supernate. Washed the RNA pellet once with 75% ethanol (at least 1 mL), mixed the sample by vortexing and centrifuged at 7,500 x g for 5 min at 4°C.
- Redissolving: dried the RNA pellet 10 min, dissolved RNA in RNase-free water. RNA solution should have an A260/280 ratio < 1.6.

Synthesis of the first-strand cDNA

SuperScript® III First-Strand Kit (Invitrogen™; Faraday Avenue, Carlsbad, California) was used. cDNA synthesis was performed in the first step from total RNA using random primers using the following conditions:

- Denature at 65°C for 5 min then place on ice for at least 1 min.
- Anneal at 25°C for 10 min.
- cDNA synthesis at 50°C for 50 min.
- Terminate reaction at 85°C for 5 min.
- RNA was removed by added RNase H then incubated at 37°C for 20 min.

The synthesized cDNA could be stored at -20°C or used for real-time PCR (LightCycler® 480 SYBR Green I Master; Roch, Oregon, USA) immediately.

Real-time PCR

The amount of COX-2 mRNA was quantified by real-time PCR in a LightCycler® 480 System using predesigned validated assays.

- RT-PCR mixture consisted of a heat-Taq polymerase, deoxynucleotides and buffer (cybr green; LightCycler® 480 SYBR Green I Master, Roche, California, USA.), specific primers for COX-2 gene. Sequences of the specific primers were: forward 5'-CAG TCA AAG ATA CTC AGG CAGA-3' and reverse 5'-GCC ACT CAA GTG TTG CAC A-3'.
- The cDNA was denatured at more than 95°C for 10 min.
- Real-time PCR was performed in 45 cycles. Condition for amplification was 95°C for 10 sec, 60 °C for 10 sec and 72°C for 10 sec.

- The data were analyzed for relative gene expression using the ΔΔCt method. Gene expression data were normalized by β-actin used as endogenous control and untreated cells as calibrator.