CHAPTER 3

RESULTS

3.1. An *in vitro* model for the exposure of air pollutants

3.1.1. The construction of the exposure chamber

To get an appropriate exposure chamber, we constructed two generations of exposure chamber.

Exposure chamber generation I

Graphic model of an exposure generation I was shown in figure 3.1. The temperature in chamber was controlled by water bath. The detail of each parts will be explained in the next session.

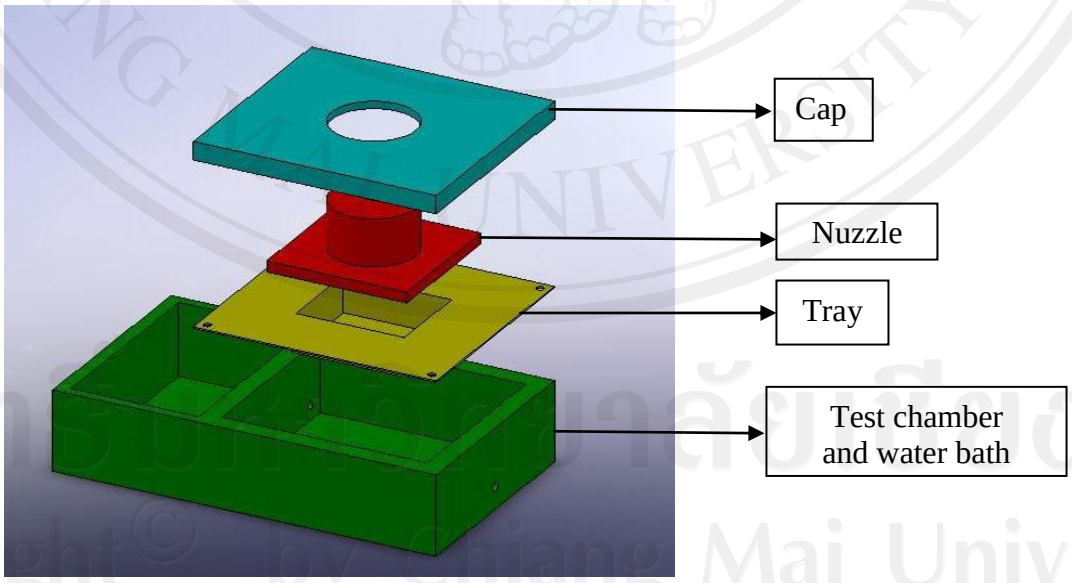
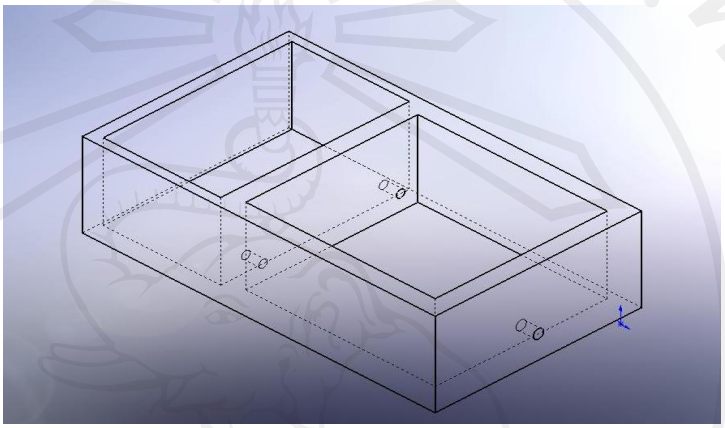


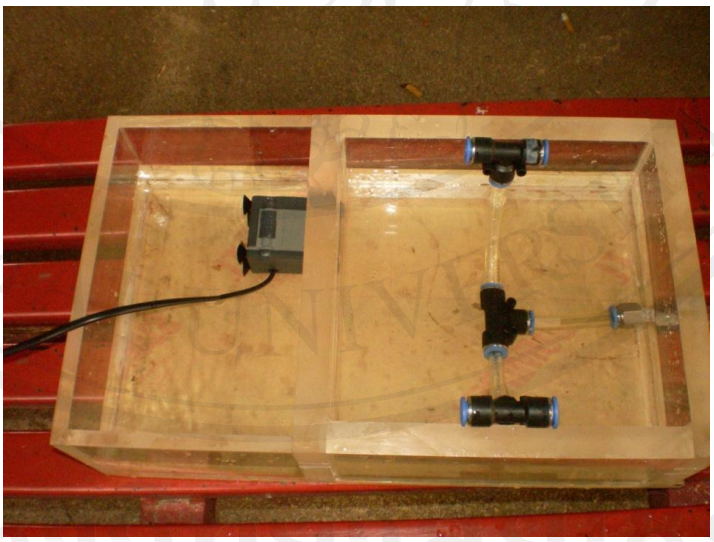
Figure 3.1 Model of exposure chamber generation 1 which contained test chamber and water bath, tray for cell culture plate, nuzzle and cap.

Test chamber

The test chamber composted of water bath and exposure box. The temperature was controlled by water bath with the sensor controller building inside this box. This chamber was connected to tested air which would be aspirated in by a pump.



(a)



(b)

Figure 3.2 Graphic model of test chamber (a) and an actual test chamber which made by acrylic (b)

Cell culture tray

It was made by stainless. The cell culture tray needed to be sterilized by UV light before testing. Before testing the air from outside chamber, this part was evacuated by aspiring the inside air out. Thus, each corner of this tray was drilled to let the air pass through.

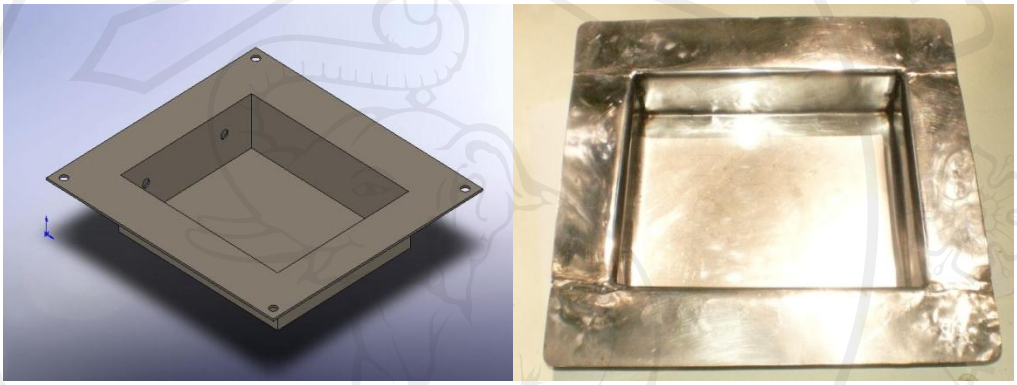


Figure 3.3 Cell culture tray

Air cap and nozzle

Cap of the testing chamber is connected to the test chamber air filtration system to prevent contamination during testing. The cover is designed for head shaped like a horn to be able to spread out the air to cover the whole exposure area. The air nozzle is designed into different shapes.

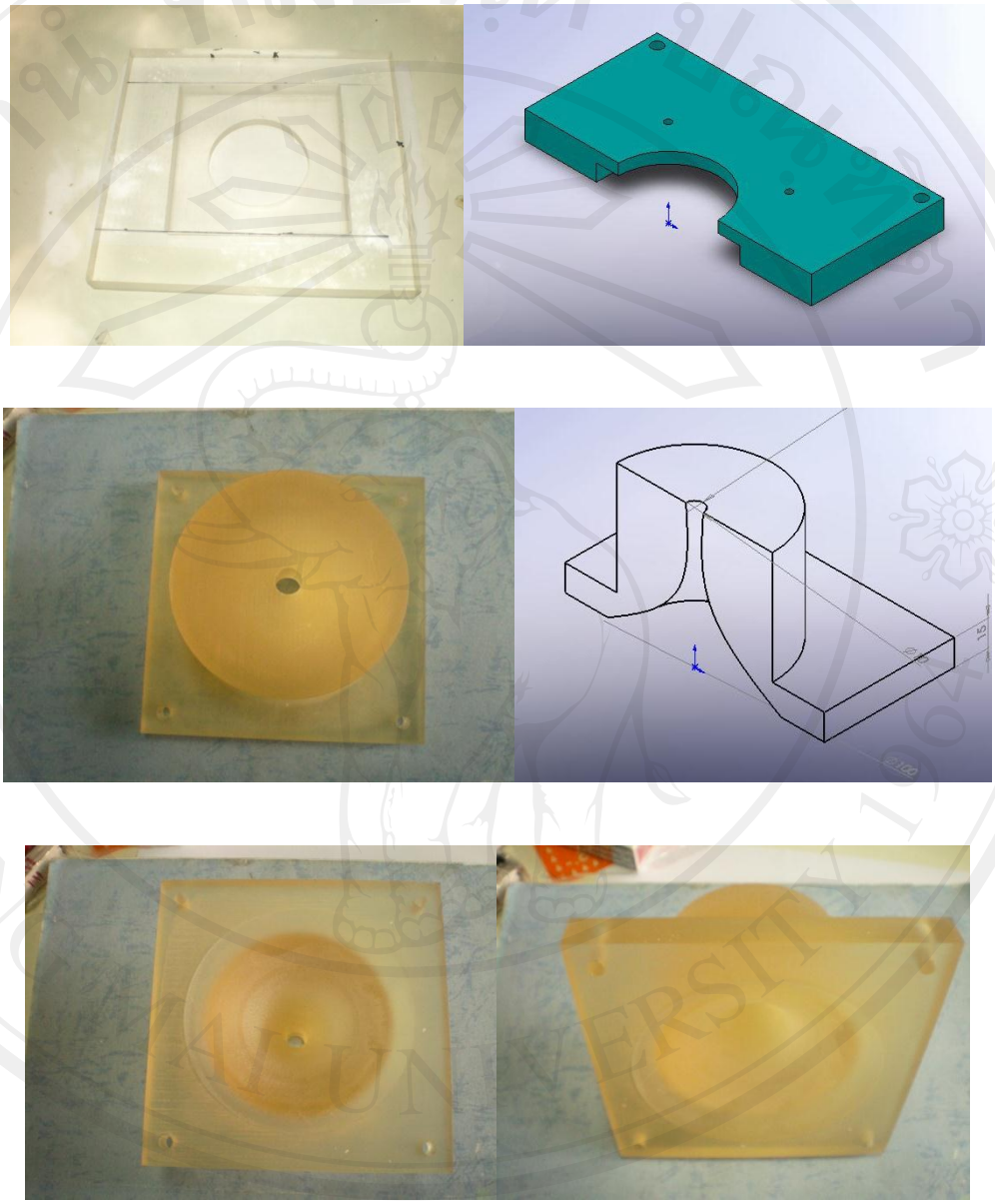


Figure 3.4 Air cap and nozzle

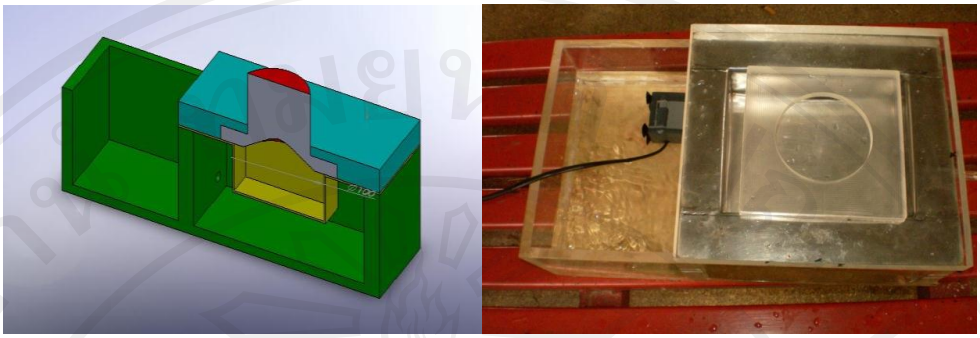


Figure 3.5 Exposure chamber generation 1

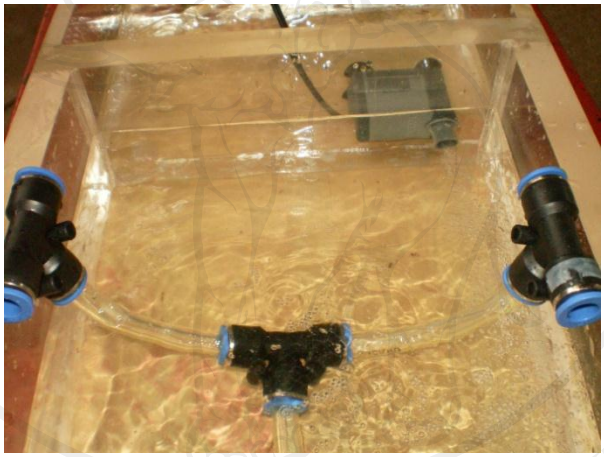


Figure 3.6 The temperature was controlled by water bath.

The exposure chamber generation I can be used to study the toxicity of air pollution. However, using water bath as a temperature controller made this chamber very big and heavy. It was very difficult to carry it to the tested sites.

Exposure chamber generation II

Temperature in this chamber was controlled by the heater. The cultures on the insert were run as conventional submersed cultures plates at 37°C in an incubator (5%

CO_2, 100% humidity) and used in the exposure chamber system. Here cells are only immersed in medium, the apical medium being removed; they were maintained and exposed to the air/liquid interface until the end of the experiment. Each exposure chamber could insert cultures plates for cell cultures maintained at 37°C by a regulated flow of temperature-controlled air in the inner space of the chamber. The exposure chamber allowed the cells in the air/liquid interface to be exposed to different mixtures of gases or particles via negative pressure through the module. The gases or particles entered by a trumpet-shaped inlet leaving each well from corresponding holes, guaranteeing an equal distribution of the gases in the three wells and on the entire surface of the culture. We aimed to construct an exposure chamber that could mimic the human lung. Each part of the exposure chamber is shown in Figure.3.7 and Figure.3.8.

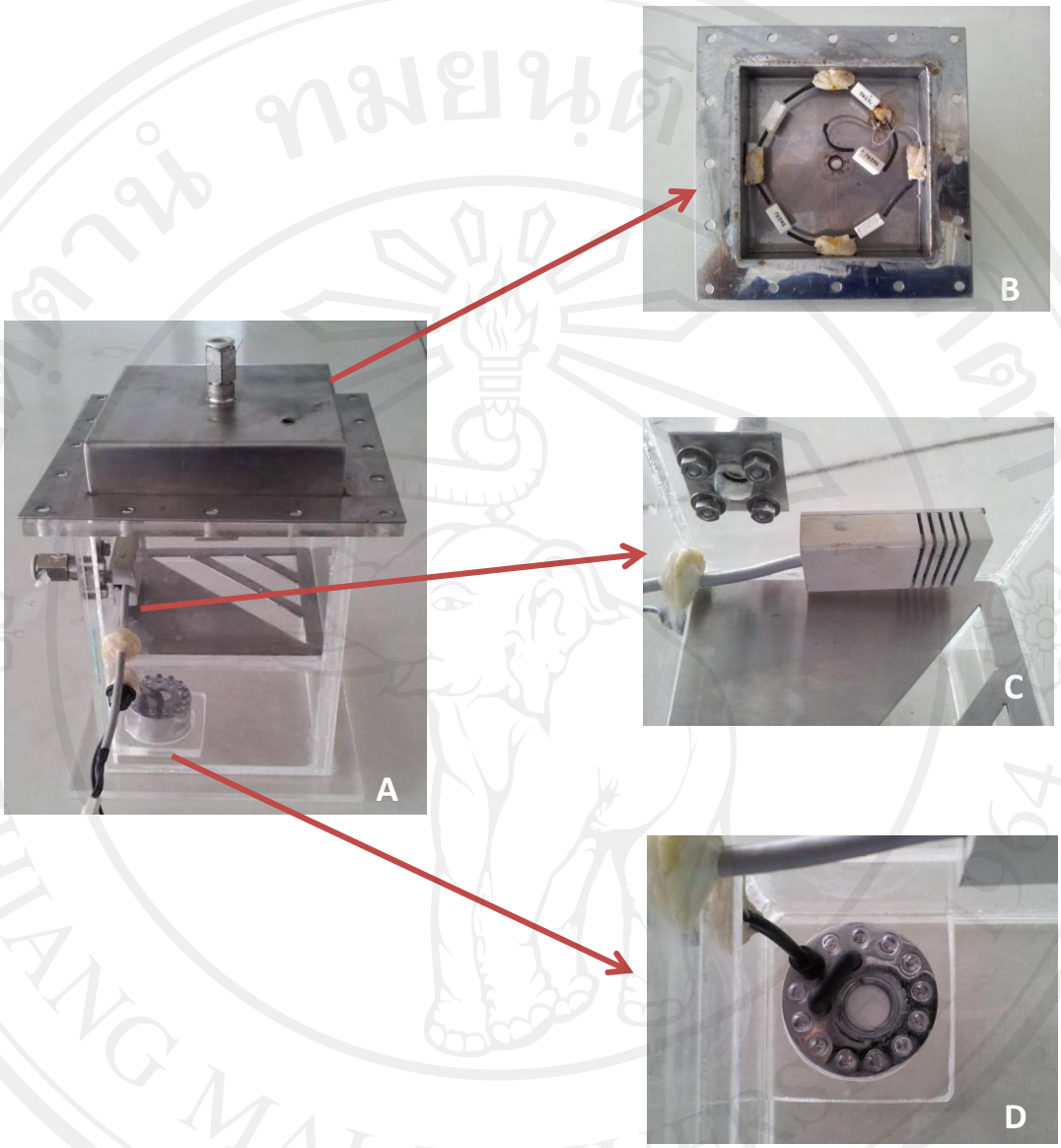


Figure 3.7 An exposure chamber device. The culture zone (chamber) (A) including Heater (B), Temperature and Humidity sensor (C) and Ultrasonic Humidifier (D).

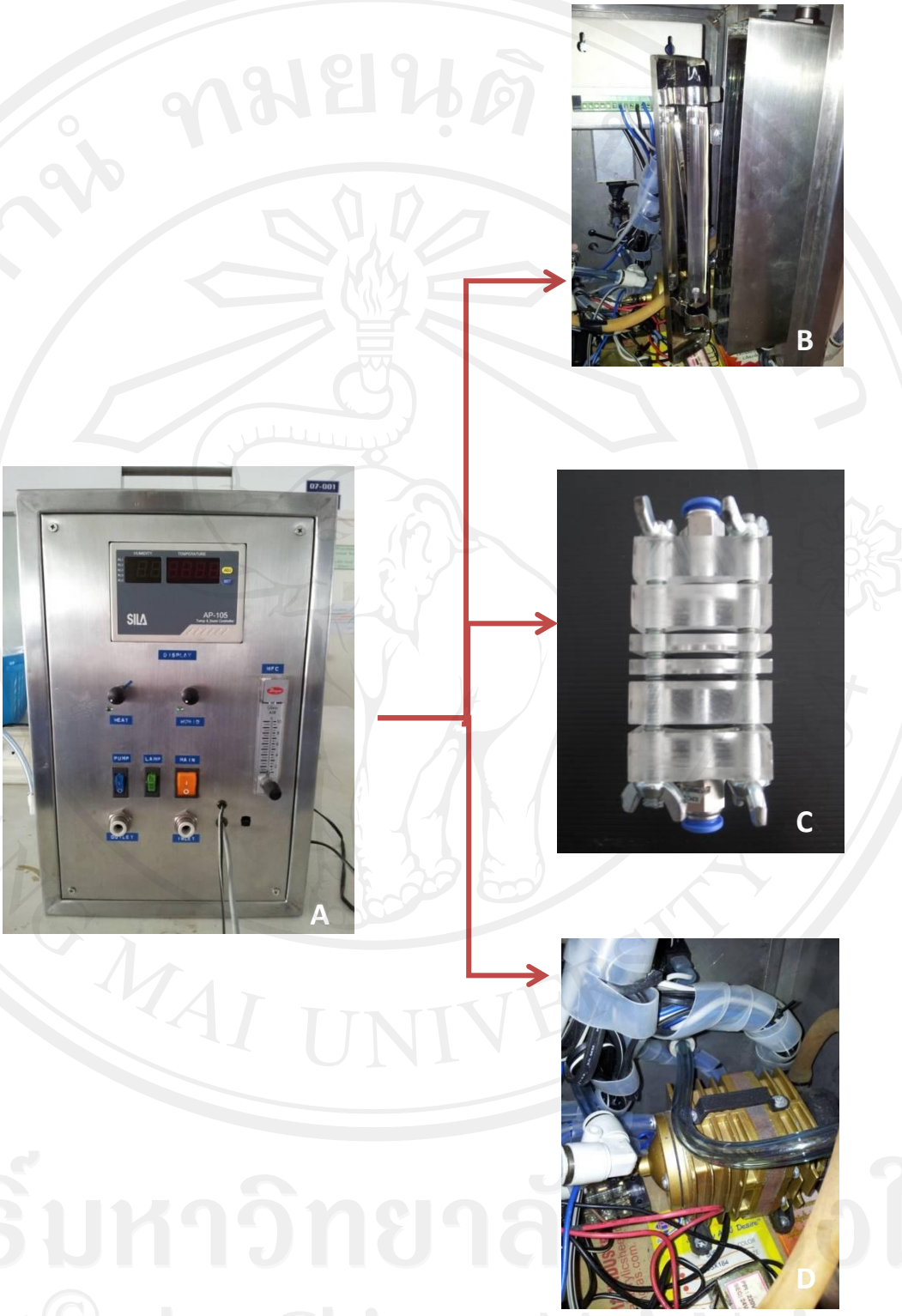


Figure 3.8 The exposure chamber control devices (A) including UV light (B), impactor (C), and pump (D).

3.1.2. Tested function of an exposure chamber

3.1.2.1. Flow rate testing

We calibrated the rate of air flow into the chamber to between 0-120 min after opening the device. The flow rate was measured and adjusted via a flow meter (data shown in Table 3.1). We adjusted the flow rate of air to 5 L/min up to 120 min; a rate equal to human breathing. Air was distributed equally in the culture zone.

3.1.2.2. Temperature testing

We monitored the temperature in the chamber from 0-120 min after opening the device. The temperature was adjusted to 37◦C starting from room temperature (Table 3.1).

3.1.2.3. Humidity testing

We measured the humidity in the chamber using a humidity meter (Table 3.1). We found that the humidity in the chamber was near 99% RH at room temperature but decreased to 60%RH at the experimental temperature (37◦C). This result indicated that the humidity depends on temperature. We found this did not affect cell viability.

Table 3.1 Summary data of Air Exposure chamber tests. Values indicate 120 min after opening the chamber.

Exposure chamber NO.	Temperature(◦C)	Humidity (%)	Flow rate (L/min)
NO.1	33.9±2.4	69.6±8.0	5
NO.2	32.3±2.1	75.1±6.5	5

3.1.2.4. The calculation of impactor for separated PM 2.5 in the air

PM 2.5 in the air was separated by impactor for the experiments. The impactor was designed based on the Stokes' theory [84] as explained below .

Impaction is a special case of curvilinear motion that finds extensive application in collection and measurement of aerosol particles. All inertial inpactors operate on the same principle. As shown in Figure 3.3, an aerosol is passed through a nozzle and the output stream (jet) direct against a flat plate. The flat plate, called an *impaction plate*, deflects the flow to form an abrupt 90◦ bend in the streamlines. Particles whose inertia exceeds a certain value are unable to follow the streamlines and collide (impact) on the flat plate. For the moment, we assume that the particles stick to the surface if they hit it. Smaller particles can follow the streamlines and avoid hitting the impaction plate. They remain airborne and flow out of the impactor. Thus, an impactor separates aerosol particles into two size ranges; particle larger than a certain aerodynamic size are removed from the air stream, and those smaller than that size remain airborne and pass through the inpactor.

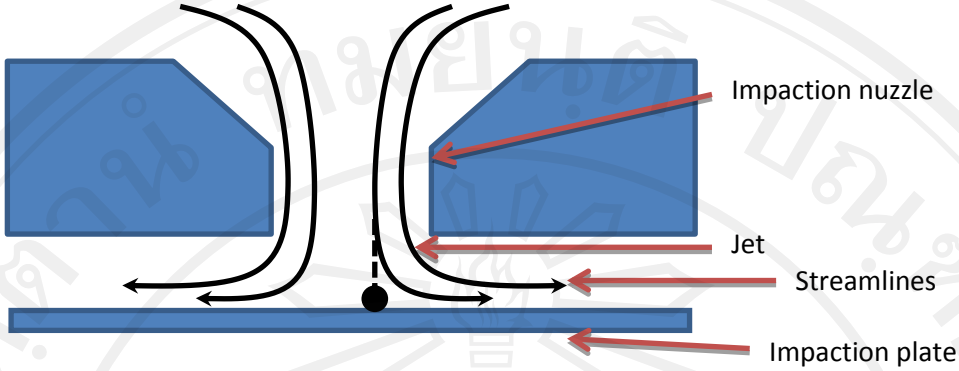


Figure 3.9 Cross-sectional view of an impactor.

In this study, we calculated the 50% cut off or d_{50} for impactor separated PM 2.5. The 50% cut-off size, d50 means that 50% of cut-off-size particles penetrate through the impactor and the other 50% are collected on the impaction plate. The d50 can be found using the following equation.

$$d_{50} = \left(\frac{9\mu W(Stk_{50})}{\rho_p V_0 C}\right)^{1/2}$$

where μ is air viscosity, W is the width or diameter of the impactor nozzle, $Stk50$ is the Stokes number corresponding to a 50% particle cut-off, ρ_p is the particle density, V_0 is average air velocity in the nozzle, and C is the size-dependent Cunningham slip correction factor.

3.1.2.5. Sterility tests

A549 cells were cultured by conventional method and put into the chamber. Cells were exposure to the air by using an exposure chamber for 24 h. The contamination by bacteria was observed under microscope. We found no contamination by bacteria. This indicated that the UV lamp could destroy contaminating bacteria from the air.

3.2. The possibility of using an exposure chamber to culture A549 cells

In this process A549 cells were cultured by conventional culture methods and placed in the exposure chamber. After that we turned on the pump and air was allowed into contact with the cells for 24 h. After exposure to the air for 24 h, cell viability and cell morphology were assayed (Figure 3.10 and Figure 3.11). No changes in viability or morphology were observed, indicating that we could use this exposure chamber for toxicity testing of ambient air pollution.

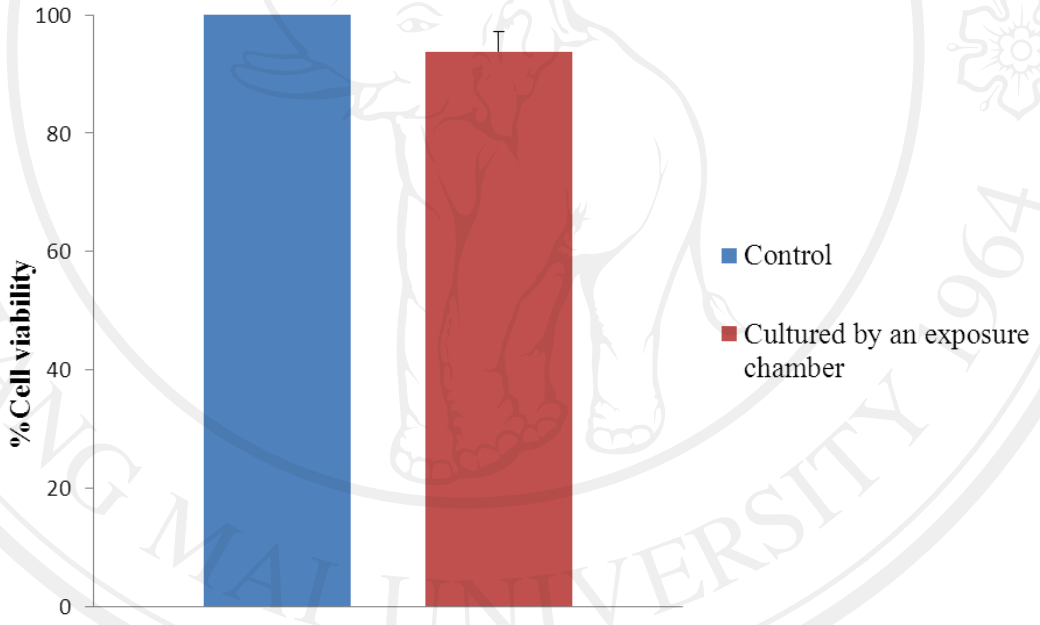


Figure 3.10 Cell viability of A549 cells after culture in an exposure chamber for 24 h. Cell viability was not significantly different from control.

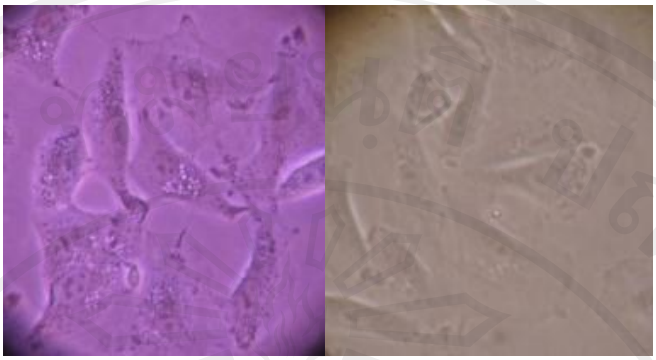


Figure 3.11 The morphology of A549 cells after culture in an exposure chamber for 24 h.

3.3. The suitable cell culture technique for toxicity testing of inhaled substances

Lung epithelial cells are the first targets of exposure to inhaled substances. Human lung epithelial cells line the alveolar sac. The apical side of the cells is directly exposed to inhaled substances, while the basolateral side is attached to the blood vessel. An air/liquid interface culture is comparable to human epithelial cells. Conventional culture methods are not suitable for toxicity testing of inhaled substances because of the barrier formed by the culture medium. In this study, A549 cells cultured by an air/liquid interface showed more response to the vapor of benzene than did conventional culture. Cell viability of the benzene exposed cells cultured by an air/liquid interface technique was only 30% when compared with unexposed control. In the conventional cell culture, the viability of benzene exposed cells was about 65% as shown in Figure 3.12.

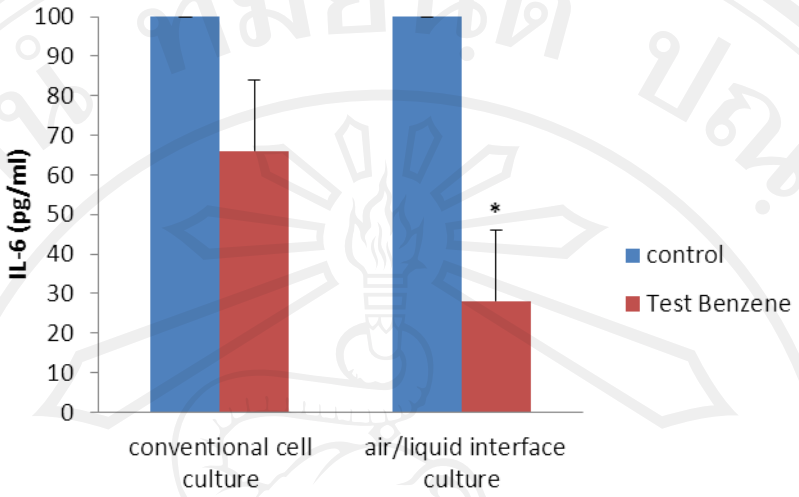


Figure 3.12 Cytotoxic effects of benzene vapor in A549 cells cultured using conventional and air/liquid interface culture techniques. Significance of differences is indicated by * for $p<0.05$. Control: % cell viability=100.

Previous results indicated that cells cultured by an air/liquid interface technique were more responsive to inhaled toxic substances. Thus the data from conventional cell cultures might not reflect the real level of toxicity in humans. We tested this hypothesis by determining the levels of IL-6 in cell culture supernatants. Cells cultured by both methods were exposed to a vapor of benzene for 30 minutes. This dose of exposure did not cause any cytotoxicity to the cells. The level of IL-6 in cells cultured by an air/liquid interface was significantly difference from non exposure control. We did not observe any differences on a conventional cell culture as shown in Figure 3.13. This data indicated that the suitable cell culture technique for toxicity testing of inhaled substances was an air/liquid interface technique.

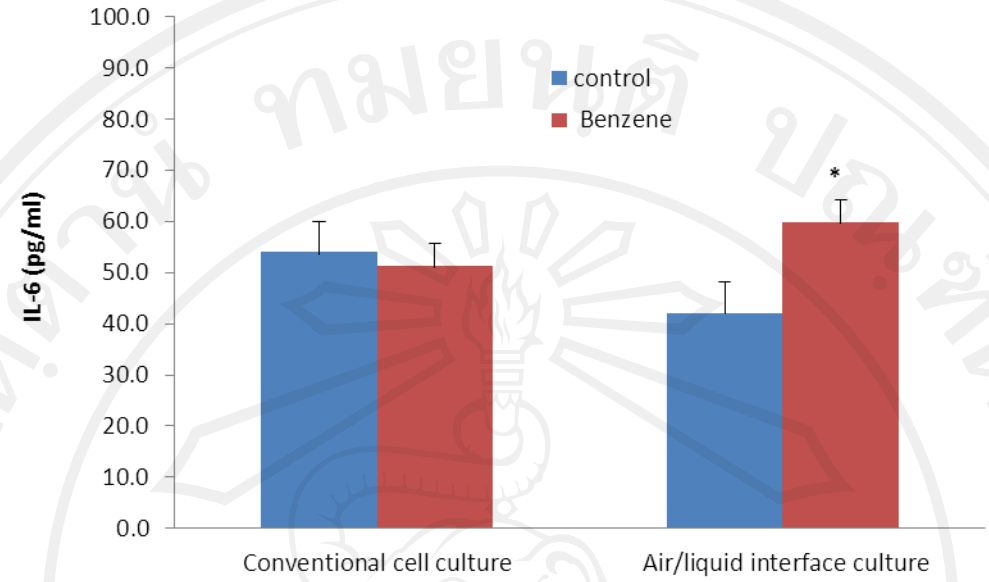


Figure 3.13 Effect of benzene vapor on IL-6 level. A549 cells were cultured using both conventional and air/liquid interface techniques. Cells were exposed to a vapor of benzene for 30 min. Culture supernatants were analyzed for IL-6 levels by an ELISA assay. Significance of differences is indicated by * for $p < 0.05$.

3.4. Toxicity testing of air pollution by using the Developed Direct Alveolar Epithelial Cells (A549)-Air Exposure Prototype

Previous result indicated that cells that cultured by an air/liquid interface technique were more response to inhaled toxic substances and indicated that we can use this exposure chamber for toxicity testing of ambient air pollution. Next, we used the Air Exposure chamber to test on actual pollution sources. We selected three sources which are divided into two groups.

1. Test site 2 regions, were Warorot market and Saraphi District
2. Control site was Phalad temple Ampher Muang, Chiang Mai.

3.4.1. Cytotoxicity of A549 cells after exposure to PM2.5 with the Air Exposure Chamber.

Under the experimental conditions, none of PM from three sources induced a decrease in the viability and the viability not differences when compare between three sources evaluated by XTT assay (Figure 3.14). This result indicated that PM concentration in each sites less than LC50 did not cause any cytotoxicity to the cells. However, we can compare IL-6 and IL-8 secretion between three sources because did not effect from cells dead.

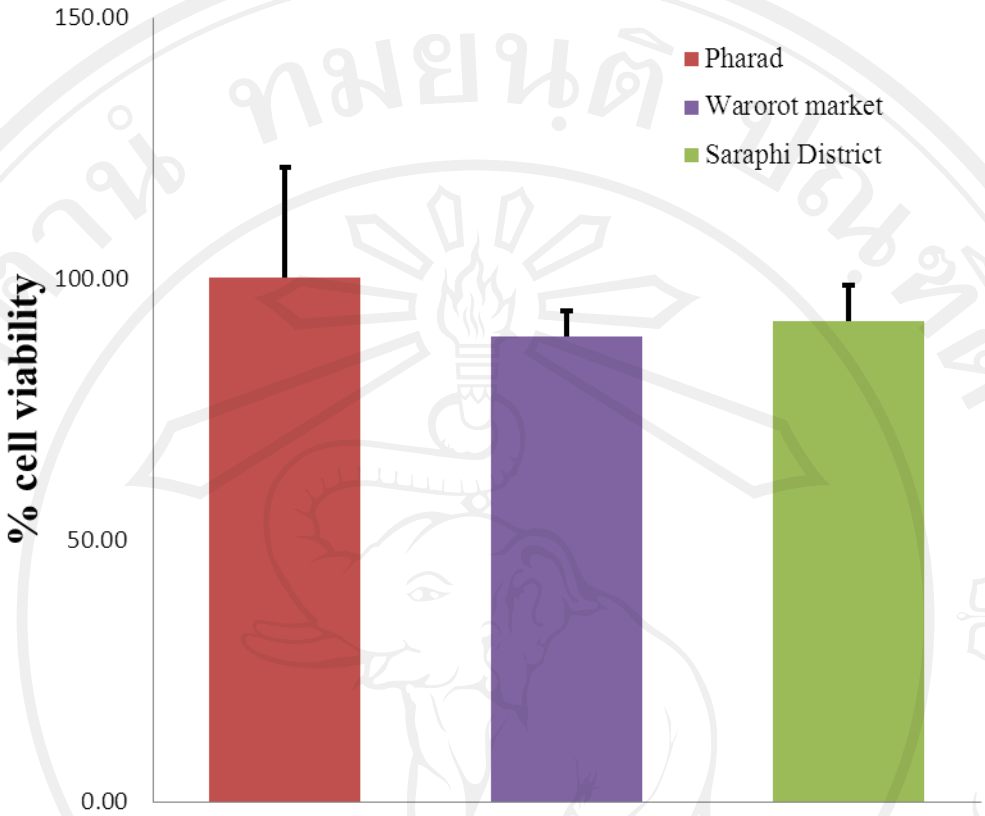


Figure 3.14 Cell viability of A549 cells after culture in an exposure chamber for 6 h. Cell viability was not significantly different from controls. Control: % cell viability is equal to100.

3.4.2. IL-6 and IL-8 secretion by A549 cells after exposure to PM2.5 with the Air Exposure Chamber.

The effect of PM2.5 on IL-6 and IL-8 levels in A549 cells were measured using the air/liquid interface technique. Cells were exposed to PM from Phalad temple, Warorot market and Saraphi using the air Exposure Chamber for 6 h. Culture supernatants were analyzed for IL-6 and IL-8 levels by an ELISA assay. Both cytokine levels (IL-6 and IL-8) in cells exposed air from Phalad temple were not significantly different from controls that were cultured in the incubator (37◦C, 5% CO_2). This result indicated that we could use this site as a control site. Previous study indicated that PM from this site did not cause DNA damage in A549 cells [(83)]. For test sites, air pollution from Warorot market caused a decrease in the secretion of IL-6 and the air pollution from Saraphi district caused an increase in the secretion of IL-6 but not IL-8 levels as shown in Figure 3.15 and Figure 3.16.

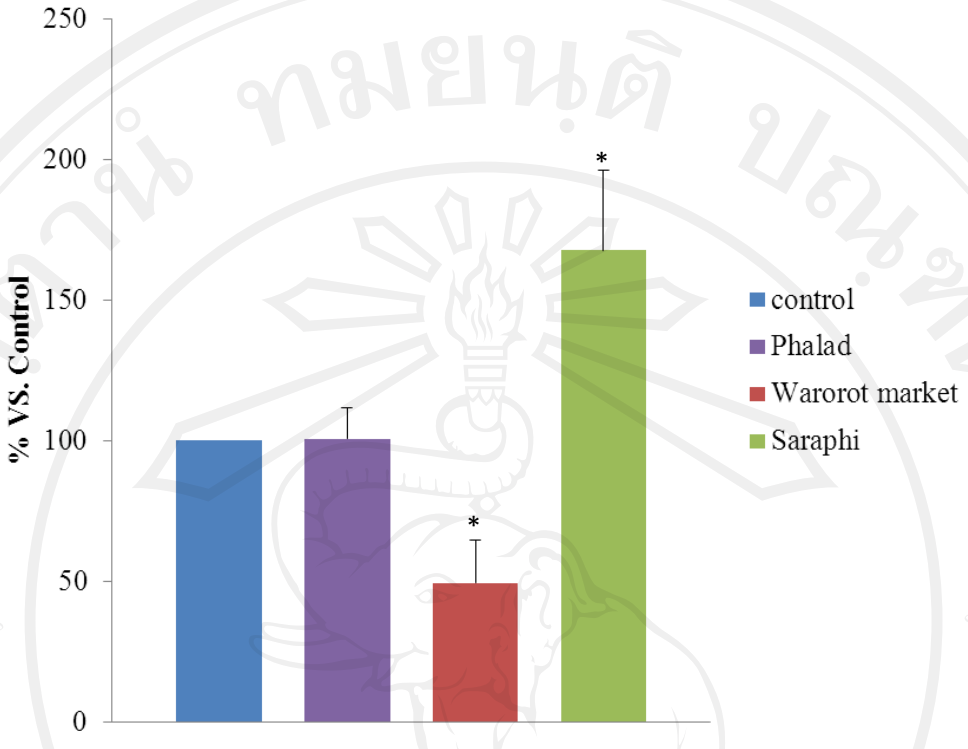


Figure 3.15 Effect of PM on IL-6 level. A549 cells were cultured using the air/liquid interface technique. Cells were exposed to PM from Phalad temple, Warorot market and Saraphi for 6 h. Culture supernatants were analyzed for IL-6 levels by an ELISA assay.

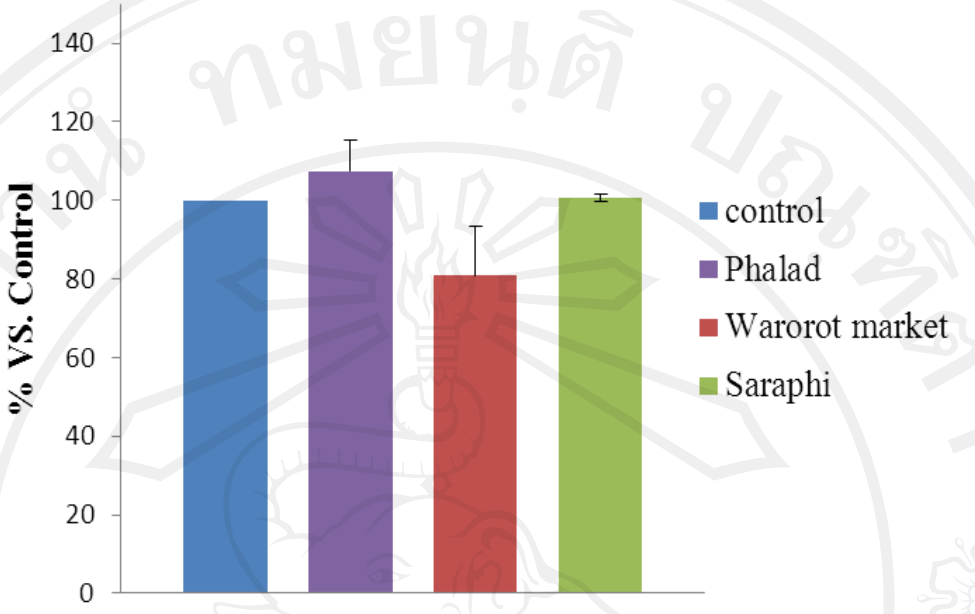


Figure 3.16 Effect of PM on IL-8 level. A549 cells were cultured using the air/liquid interface technique. Cells were exposed to PM from Phalad temple, Warorot market and Saraphi for 6 h. Culture supernatants were analyzed for IL-8 levels by an ELISA assay.

Since we found no change of IL-8 as we expected, the culture media were collected both immediately after 6h exposure and 24 h after continued culture in CO_2 incubator. The result of IL-6 and IL-8 levels for Phalad temple were still not significantly different from controls that were cultured in the incubator (37◦C, 5% CO_2) as shown in Figure.3.17 and Figure 3.18.

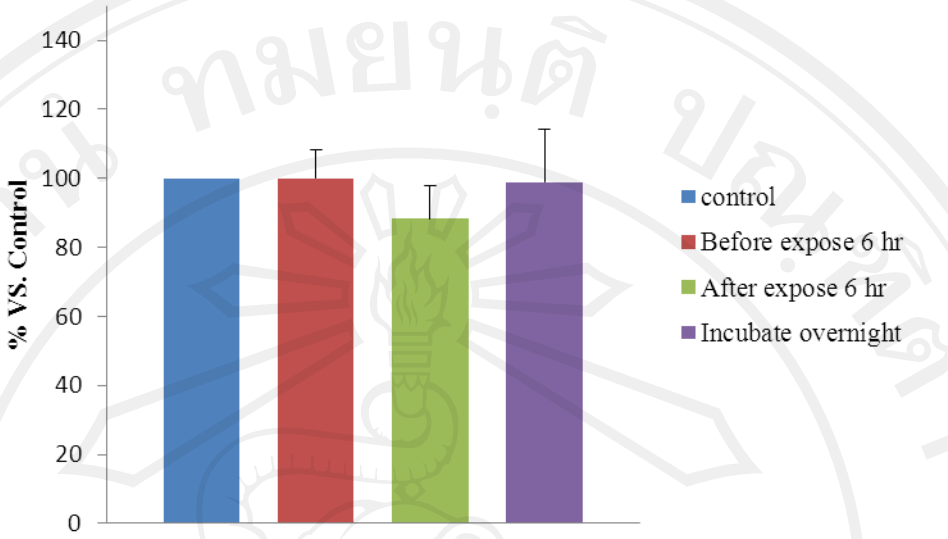


Figure 3.17 Effect of PM on IL-6 level. A549 cells were cultured using the air/liquid interface technique. Cells were exposed to PM at Phalad temple for 6 h. Culture supernatants were analyzed for IL-6 levels by an ELISA assay.

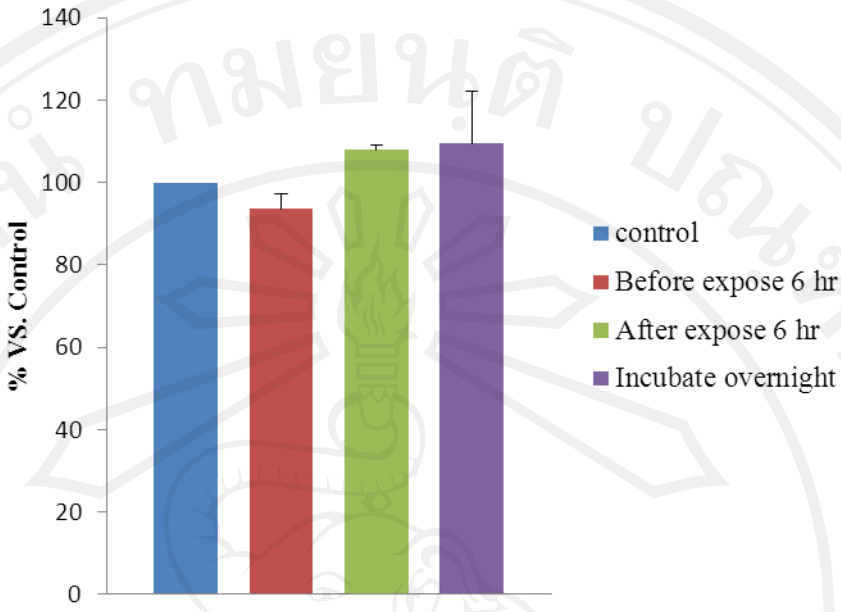


Figure 3.18 Effect of PM on IL-8 level. A549 cells were cultured using the air/liquid interface technique. Cells were exposed to PM at Phalad temple for 6 h. Culture supernatants were analyzed for IL-8 levels by an ELISA assay.

The above procedure was repeated at Warorot market (Figure 3.19 and Figure 3.20). After 6h of cells exposure, IL-6 level was decreased but IL-8 level was increased.

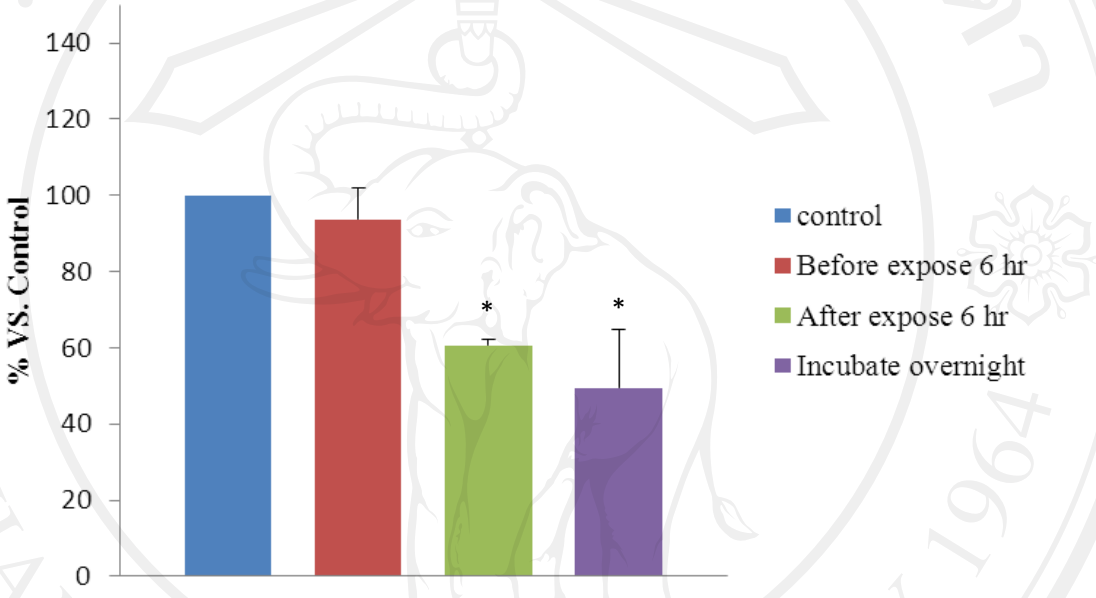


Figure 3.19 Effect of PM on IL-6 level. A549 cells were cultured using the air/liquid interface technique. Cells were exposed to PM at Warorot market for 6 h. Culture supernatants were analyzed for IL-6 levels by an ELISA assay. Significance of differences is indicated by * for $p<0.05$ (n=3).

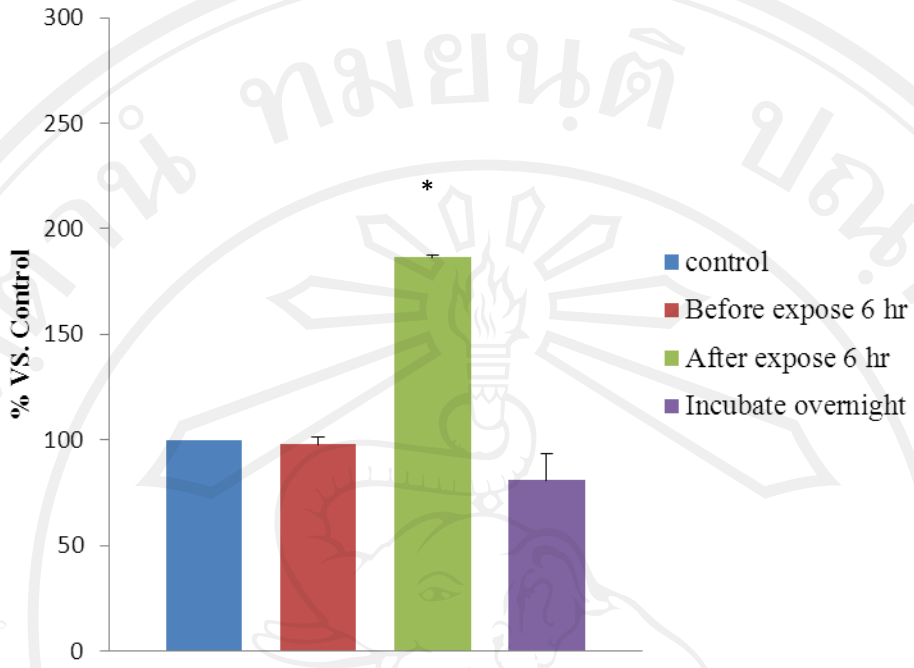


Figure 3.20 Effect of PM on IL-8 level. A549 cells were culture using an air/liquid interface technique. Cells were exposed to a PM of Warorot market for 6 h. Culture supernatant were determined the IL-8 level by an ELISA assay. Significance of differences is indicated by * for $p<0.05$ (n=3).

The same experiment was done at Saraphi District (Figure 3.21 and Figure 3.22). Air pollution from Saraphi District induced an increase in the secretion of IL-6 and IL-8 in culture media after 6h exposure.

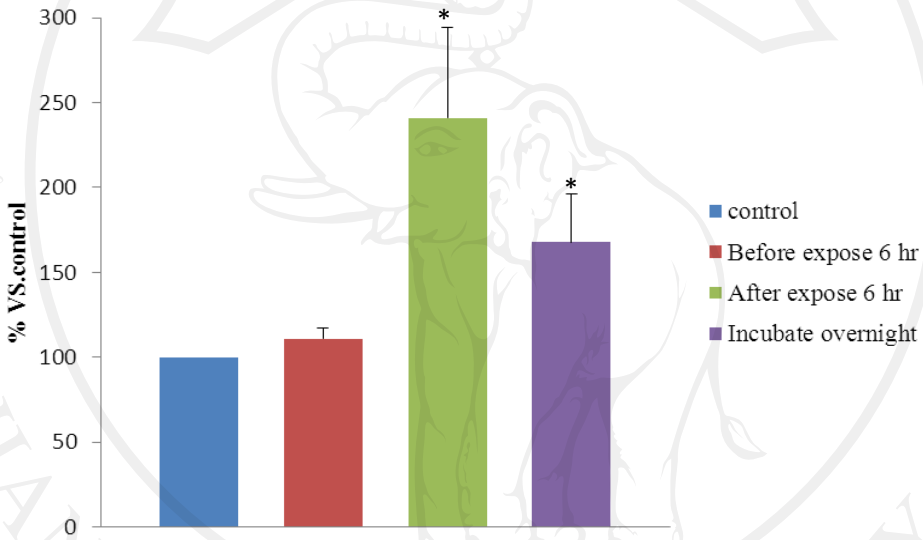


Figure 3.21 Effect of PM on IL-6 level. A549 cells were cultured using the air/liquid interface technique. Cells were exposed to PM at Saraphi for 6 h. Culture supernatants were analyzed for IL-6 levels by an ELISA assay. Significance of differences is indicated by * for $p<0.05$ (n=3).

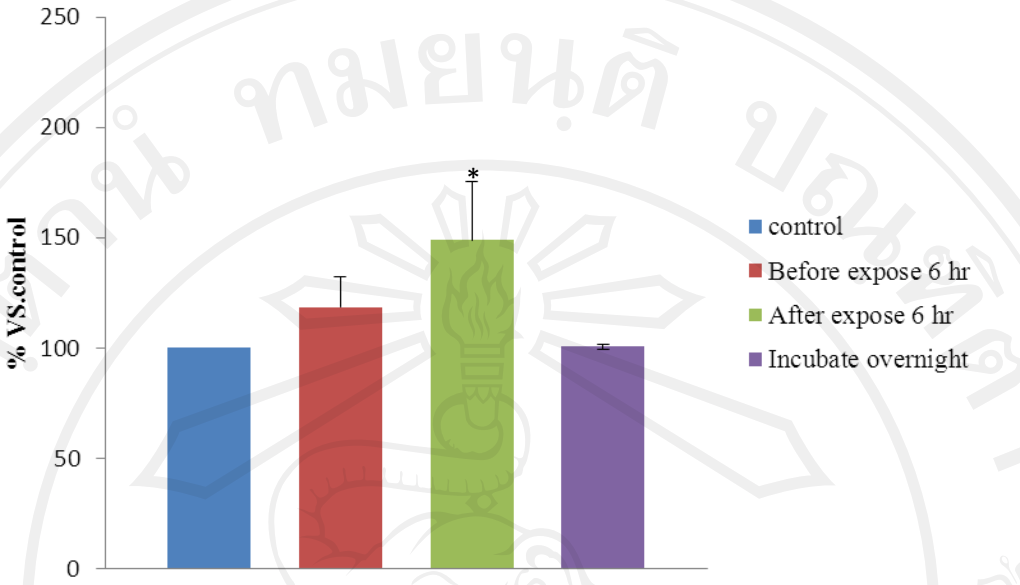


Figure 3.22 Effect of PM on IL-8 level. A549 cells were cultured using the air/liquid interface technique. Cells were exposed to PM at Saraphi for 6 h. Culture supernatants were analyzed for IL-8 levels by an ELISA assay. Significance of differences is indicated by * for $p<0.05$.(n=2) Control : %VS .control =100(n=3).

Comparison of daily PM levels demonstrated a correlation in the amounts of IL-6 and IL-8 secreted from cells and PM level at Saraphi, but not at Warorot market (Figure 3.23). However our data contained only 3 experiments, more data should be collected to evaluate whether the level of PM is actually correlate to the changing of cytokine levels.

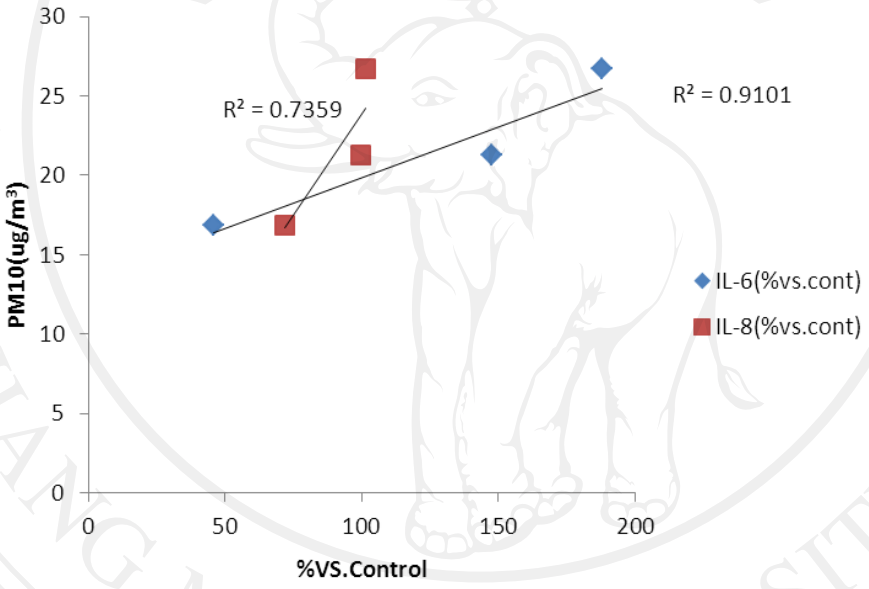


Figure 3.23 Correlation of daily PM with effect of PM on IL-6 and IL-8 levels at Saraphi. Cells were exposed to PM for 6 h. Culture supernatants were analyzed for the IL-6 and IL-8 level by an ELISA assay.

3.5 IL-6 and IL-8 secretion by A549 cells after exposure to PM2.5 with Conventional method.

In conventional method, PM2.5 was collected onto membrane, extracted and cultured with A549 cells. In all tested sites, PM2.5 did not have any toxic to A549 cells. Cell viability and the levels of IL-6 and IL-8 were not differences compared to untreated cells. The results were shown in Figure 3.24, Figure 3.25, and Figure 3.26.

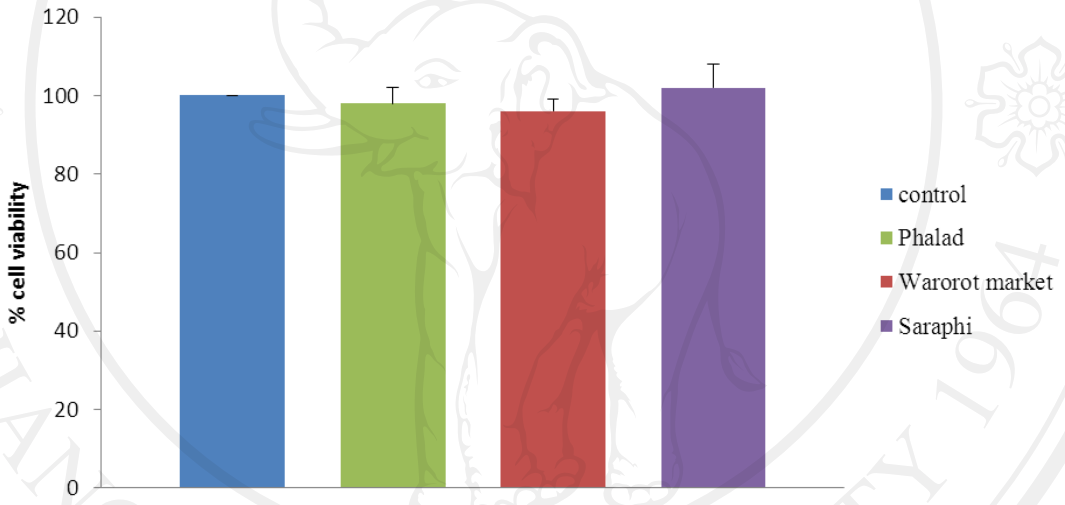


Figure 3.24 Cell viability of A549 after exposure to PM2.5 with conventional method. Cell viability was not significantly different from controls.

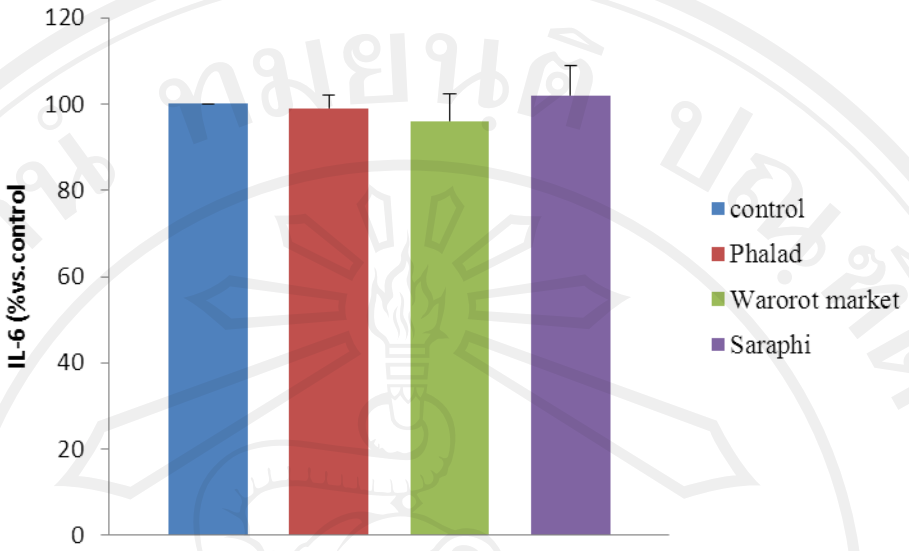


Figure 3.25 Effect of PM on IL-6 level. A549 cells after exposure to PM2.5 with conventional method. Culture supernatants were analyzed for IL-6 levels by an ELISA assay.

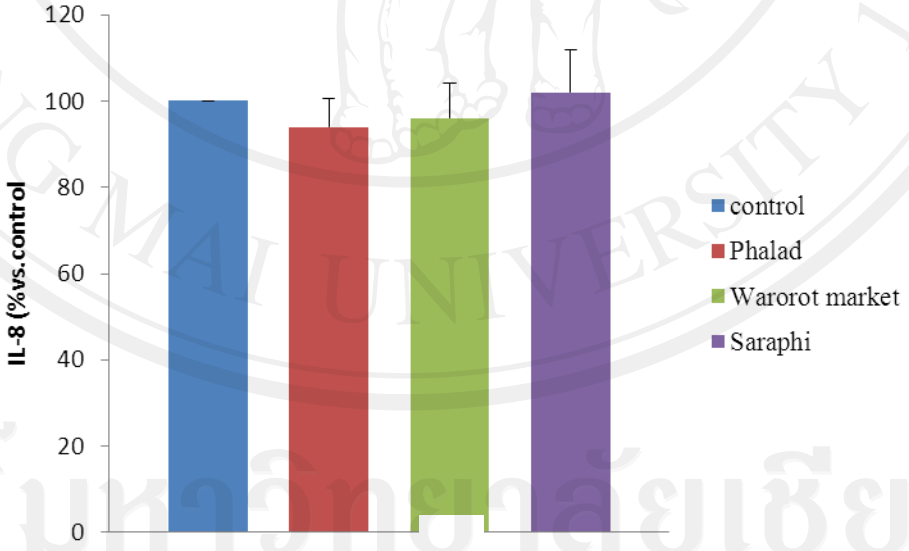


Figure 3.26 Effect of PM on IL-8 level. A549 cells after exposure to PM2.5 with Conventional method. Culture supernatants were analyzed for IL-8 levels by an ELISA assay.