ORIGINAL ARTICLE

Mammalian Lung Cell Cytotoxicity and Genotoxicity Evaluation of Organic and Inorganic PM_1 and $PM_{0.1}$ of Urban Air at UKM, Malaysia

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ABSTRACT

Introduction: Particulate matter (PM) is one of the main causes of air pollution, and it has been demonstrated to pose significant health risk to human. However, the cytotoxicity and genotoxicity of the fine (PM_1) and ultrafine particles ($PM_{0.1}$) are yet to be confirmed. Hence, this study aimed to determine the genotoxicity of the organic and inorganic extracts from the PM_1 and $PM_{0.1}$ against the V79 Chinese hamster mammalian lung cells. **Methods:** In this study, air sampling was conducted by using the Nanosampler Model 3180 in Universiti Kebangsaan Malaysia (UKM), Kuala Lumpur, Malaysia. Then, the organic and inorganic extracts of PM_1 and $PM_{0.1}$ were subjected to cytotoxicity and genotoxicity testing by using the MTT assay and alkaline comet assay, respectively on the V79 Chinese hamster mammalian lung cell line. **Results:** The V79 Chinese hamster lung cells exhibited cytotoxic effects when exposed to extracts from both organic and inorganic sources of PM_1 and $PM_{0.1}$. Notably, PM_1 displayed greater cytotoxicity compared to $PM_{0.1}$. Interestingly, solely the organic extracts from PM_1 and $PM_{0.1}$ revealed noteworthy ($p<0.05$) genotoxic impacts on the V79 Chinese hamster lung cells, whereas the inorganic extract did not. Furthermore, the ultrafine $PM_{0.1}$ displayed higher genotoxicity in comparison to PM_1. **Conclusion:** Our current findings showed that the organic extracts of PM_1 and $PM_{0.1}$ may cause DNA damage towards the V79 Chinese hamster mammalian lung cell line, and the genotoxicity of the ultrafine $PM_{0.1}$ was greater than the PM_1.

Keywords: Air sample; Cytotoxicity; Genotoxicity; $PM_{0.1}$, PM_1

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INTRODUCTION

Decades of research have shown that air pollutants such as particulate matter (PM) and ozone increase significantly and are associated with various health problems. PM is a multifaceted combination of fine particles and liquid droplets varying in composition and size, including acids (e.g., nitrates and sulfates), organic compounds, metals, and particles of soil or dust (1). Generally, PM has been classified based on its aerodynamic diameter, and is categorized as PM_{10} (particles with aerodynamic diameter ≤ 10 μm), $PM_{2.5}$ (particles with aerodynamic diameter ≤ 2.5 μm) and PM_1 (particles with aerodynamic diameter ≤ 1 μm). Currently, the degree of PM pollution is commonly determined based on the density of $PM_{2.5}$ and PM_{10} and, to a lesser extent, PM_1. Studies about the particle size and its source-related components have been limited because chemical composition varies according to locations and climates, which are challenging to study extensively (2,3).

However, it has recently been discovered that the greatest threats to human health arising from ultrafine particulate matter ($PM_{0.1}$). $PM_{0.1}$, or also known as ultrafine particles, are nanoparticles with an aerodynamic diameter ≤ 0.1 μm or 100 nm. Information about the physiochemical, sources, composition and characteristics of $PM_{0.1}$ remains incomplete because its small size makes it challenging to study. The majority of research has indicated that inhaling $PM_{0.1}$

particles has negative health impacts (4). However, studies about $PM_{0.1}$ in tropical countries and health risks are still limited. There is a commonly held belief that $PM_{0.1}$ particles are highly toxic because of their ability to absorb toxic substances due to their large surface area (5,6). Once these particles enter the human body, they can be transported to various organs and have the potential to penetrate deeply into the circulatory system through the respiratory tract (4,7). The lungs are the first organ that will be affected when exposed to inhaled particulate matter.

Most adverse effects of particulate matter are associated with respiratory and cardiopulmonary disease. Moreover, PM may induce a broad spectrum of consequences at the cellular level such as inflammation, DNA damage, and genomic instability, thus acting as a catalyst for the emergence of cancerous processes and increasing the prevalence of respiratory, neurogenerative and neurodevelopmental disorders (8). Although the cytotoxic and genotoxic effects of PM_{10} and $PM_{2.5}$ have been reported elsewhere, the cytotoxicity and genotoxicity of the PM with smaller aerodynamic diameter remains uncertain. Hence, this study aimed to determine the genotoxicity of the organic and inorganic extracts from the PM_1 and $PM_{0.1}$ collected from the Universiti Kebangsaan Malaysia (UKM) in Kuala Lumpur, Malaysia against the V79 Chinese hamster mammalian lung cells. The capability of PM of varying sizes to prompt DNA damage was explored via the alkaline comet assay.

MATERIALS AND METHODS

Air sampling

The detailed methodology on air sampling has been reported by Jamhari et al. (9). Briefly, the samples were collected between 17 February 2017 and 3 December 2017, and pooled samples were used for cytotoxicity and genotoxicity assessment in this study. The sampling was representative of a typical tropical urban air near the main road with high traffic. The air samples were collected at the rooftop (about 15 m above ground level) of the UKM Kuala Lumpur campus building located on the road side (10 m) of Jalan Raja Muda Abdul Aziz (30 10′ 7.931″ N, 1010 42′ 4.343″ E). The traffic flow around this area was congested most of the time, especially in the morning and late afternoon, because it was surrounded by the main roads, which are Jalan Tunku Abdul Rahman (250 m) and Jalan Tun Razak (1.2 km), based on the number of vehicles.

The Nanosampler Model 3180 (Kanomax, Japan) was used to capture PM in the air. The inlet of the Nanosampler was of single channel type with unidirectional air flow operated at a flow rate of 40 L/min for 120 hours (9). The experimental set-up at the sampling sites had the impactor and pump housed in a steel electrical cabinet, with an inlet at the top of the housing covered by a rain hood. The Nanosampler 3180 was installed with PM_{10}, $PM_{2.5}$, PM_1, $PM_{0.5}$ and $PM_{0.1}$ 55 mm quartz fibrous filter (Pallflex 2500QAT-UP, Nihon Pall Ltd., Japan) and an inertial filter containing SUS stainless steel fibre wool (9.8 ± 0.03 mg) (felt type, SUS-304, Nippon Seisen Co. Ltd., Japan) supplied by the manufacturer (10). The sampling set-up for this study is demonstrated in Fig. 1. The PM of varying sizes was collected on the respective quartz fibrous filter and accumulated $PM_{0.1\ -\ 0.5}$ in the inertial filter. All filters were wrapped with aluminium foil and pre-baked at 400 °C, for 4 hours in a furnace (Carbolite Eurotherm 301 controller, UK) to eliminate possible organic residue contaminants prior to the sampling. The quartz filter paper and inertial filter were also preconditioned in a desiccator at a relative humidity of 35% ± 5% for 48 hours before and after sampling and weighed using an electronic microbalance (Sartorius, USA) with a precision reading of 0.001 mg to ensure accuracy (11). To prevent cross-contamination, each filter was individually wrapped in aluminium foil and sealed in airtight zip-lock PE bags, and all samples were kept at 4°C until the extraction of organic and inorganic compounds.

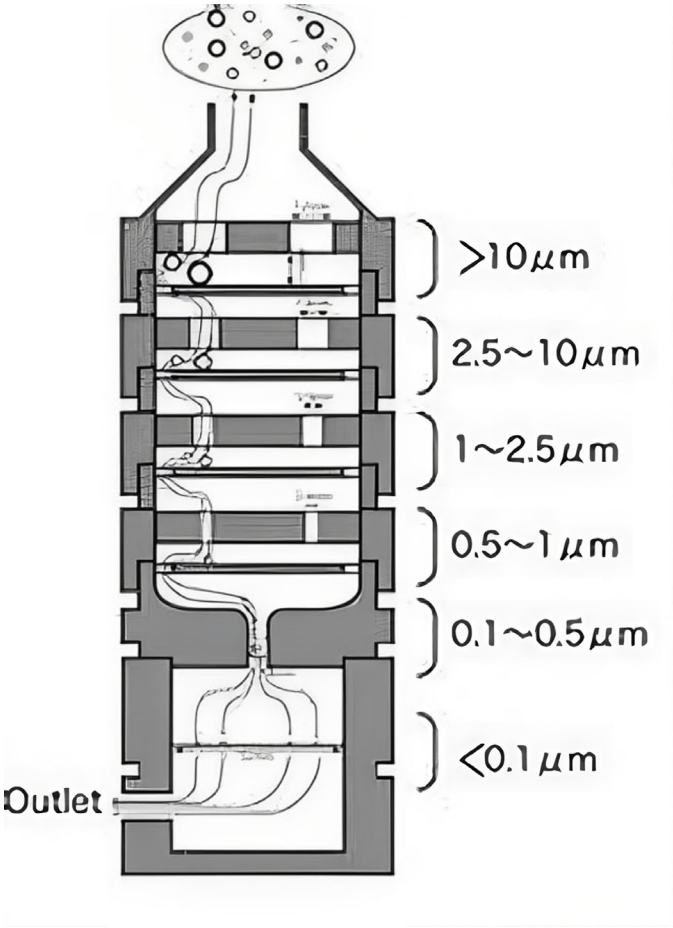

Fig. 1 : Schematic diagram of air sampling set-up using the Nanosampler 3180.

Extraction of organic and inorganic compounds

Prior to the extraction, the filters retrieved from the freezer underwent a period of equilibration to reach room temperature, and any surface moisture were eliminated through evaporation. In brief, the filter paper was cut into pieces and put in the pre-baked glassware. Subsequently, the samples were subjected to ultrasonic agitation with a mixture of dichloromethane (DCM, Merck, Germany) and n-hexane (Merck, Germany) in a 1:1 v/v ratio, in a series of 15 cycles lasting two minutes each, interspersed with one-minute intervals of rest. After two repetitions of the sonication procedure, the resulting extracts were combined. Prior to the clean-up procedure, the extracts were concentrated to approximately 200 µL utilizing a gentle nitrogen (N2) blowdown. Then, we utilized the Lichrolut® RP-18 Silica solid-phase extraction (SPE) cartridges (Merck, Germany) for the clean-up process in order to mitigate matrix interferences arising from co-extracted contaminants in the final extract. The RP-18 cartridge underwent pre-conditioning with 10 mL of n-hexane prior to being loaded with the organic sample. The desired compounds were fully eluted from the column using 10 mL of DCM:n-hexane (35:65 v/v) as the eluting solution. The organic fraction obtained was then subjected to evaporation until dryness under mild N2 flow. The dry residue was subsequently reconstituted with 500 µL of dimethyl sulfoxide (DMSO, Fisher Scientific, UK) and dissolved in 4.5 mL of Dulbecco's modified Eagles' medium (DMEM, Gibco, USA), resulting in a final DMSO concentration of less than 1% v/v for cell treatment.

Another half of each filter paper was cut into pieces and dissolved in 20 mL of DMEM without fetal bovine serum (FBS) in a 50 mL glass conical flask for the aqueous extract. This extraction process occurred over the course of 60 minutes, facilitated by an ultrasonic bath (Elmasonic S70H, Elma, Germany). Subsequently, a 10-minute centrifugation at 2500 RCF was carried out, followed by filtration through an Acrodisc® Supor® membrane filter (Pall Life Sciences, USA). For both the organic and aqueous extracts, an additional filtration step was performed using a 0.22 µm membrane filter, ensuring sterilization. The resulting $PM_{0.1}$ and PM_1 organic and inorganic extracts were then stored at -20 °C until cytotoxicity and genotoxicity analysis.

Cell line for the in-vitro assays

The V79 Chinese Hamster lung fibroblast cell line was procured from ATCC (Rockville, USA) and cultured in DMEM (Gibco, USA), as described previously (12). The cells were grown as a monolayer in a T25 cm^2 culture flask and supplemented with 1% penicillin (10000 U/mL, Gibco, USA), 10% FBS (Gibco, USA), and sodium pyruvate (PAA Laboratories, Australia) at the concentration of 0.11 mg/L. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C until they reached 70-80% confluence, after which they were harvested.

In vitro cytotoxicity assay

The viability of V79 Chinese hamster lung cells after treatment with $PM_{0.1}$ or PM_1 air sample extractsfor 24 hours was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which was slightly modified from a previous study (13). Briefly, cells were seeded in a 96-well plate at a density of $5x10^4$ cells per well in a volume of 200 µL and were treated with various concentrations of air samples. After 24 hours of exposure, 20 µL of 5mg/mL MTT solution (Sigma, USA) was added to each well, and the plate was incubated for 4 hours at 37°C. Then, the entire medium was removed, and 200 µL of DMSO (Fisher Scientific, UK) was added to dissolve formazan. The plate was further incubated for 15 minutes and followed by gentle shaking for 5 minutes to ensure the complete dissolution of formazan. The cytotoxic effects of the air samples on V79 cells were assessed by measuring the absorbance of each well using an I-MarkTM microplate reader (Bio-Rad Laboratories, USA) at 570 nm.

In vitro genotoxicity assay

The alkaline comet assay was slightly modified from the previously described methods (12,14). All the chemicals needed for preparing the lysis buffer, electrophoresis buffer, and washing buffer were purchased from Merck (Germany). Briefly, V79 cells were seeded at a density of $5x10^4$ cells/mL (2 mL per well) into six-well plates and incubated for 24 hours at 37°C in a 5% CO_2 incubator. After discarding the media, sample extracts were added at the concentration of IC_{25} for 24 hours of treatment. The negative control contained only cell media without any samples. For positive control, cells were exposed to 100 µM of hydrogen peroxide (Merck KGaA, Germany) for 30 minutes. Following treatment, the cells were washed with phosphate buffer solution (PBS, Sigma, USA) and trypsinised to remove them from the flask surface. The cells from each well were then transferred to three microcentrifuge tubes and centrifuged twice at 2,500 rpm for 5 minutes, followed by washing with PBS after each centrifugation. Frosted slides were coated with a layer of normal melting point agarose (0.6% w/v, Sigma, USA), and cells were collected and suspended in low melting point agarose (0.6% w/v, Sigma, USA) at 37°C until they solidified, before being lysed in lysis buffer (2.5M NaCl, 100 mM EDTA, 10 mM Tris, and 1% Triton-X) for 1 hour at 4°C. The slides were then placed horizontally in an electrophoresis tank and left for 20 minutes in an electrophoresis buffer (0.3N NaOH, 1 mM EDTA, pH >13) to allow DNA unwinding. Electrophoresis

was then conducted for 20 minutes at 25 V and 300 mA. After electrophoresis, slides were washed three times with neutralization buffer (400 mM Tris) before being stained with 40 µL of ethidium bromide (Sigma, USA). The slides were then analysed using a Nikon Eclipse TS-100 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a 560 nm emission filter and a 590 nm barrier filter. Tail moment and DNA tail percentage were quantified in 50 cells on each slide using the Comet Assay III program (Perceptive Instruments, UK).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 25.0 (IBM Corp, Armonk, New York, USA). The cytotoxicity and genotoxicity data were presented as mean ± standard error of the mean (SEM), and the Mann-Whitney U test was employed to determine any significant differences ($p<0.05$) between the means.

RESULTS

Cytotoxicity of air samples

The MTT assay findings showed decreased cell viability after being treated with various air samples. The IC_{25} value for organic compounds $PM_{0.1}$ and PM_1 was 42.5 µg/mL and 30.0 µg/mL, respectively. While for inorganic compounds, the IC_{25} value for $PM_{0.1}$ and PM_1 was 44.5 µg/mL and 20.5 µg/mL, respectively.

Genotoxicity of air samples

The genotoxic effects of the air samples with different sizes of PM were detected following 24 hours of treatment, based on tail moment score and DNA tail percentage. The tail moment can be calculated by multiplying the distance between the head and tail of the comet by the proportion of DNA in the tail. On the other hand, the percentage of DNA in the tail represents the proportion of DNA located in the tail region of the comet (15). The IC_{25} concentrations of various air samples, as identified via the MTT assay, were used to examine its genotoxic effects. Fig. 2 shows the tail moment scores of each sample, while Fig. 3 shows the percentage of DNA in the tail for each sample in this study. For this assay, a positive control was established using hydrogen peroxide at a concentration of 100 µM. The positive control was able to induce significant ($p<0.05$) increment in tail moment score (15.80 ± 1.24 a.u.) and percentage of DNA in tail (50.40 ± 2.69%) as compared to the negative control, which had a tail moment score of 0.31 ± 0.04 a.u. with 2.28 ± 0.30% of DNA in tail.

The tail moment score was highest in V79 cells treated with organic $PM_{0.1}$ ultrafine particles (3.13 ± 0.85 a.u.), followed by organic PM_1 fine particles (2.13 ± 0.48 a.u.), inorganic $PM_{0.1}$ ultrafine particles (2.09 ± 0.46 a.u.) and inorganic PM_1 fine particles (0.89 ± 0.17 a.u.). A similar trend of the score was observed in the

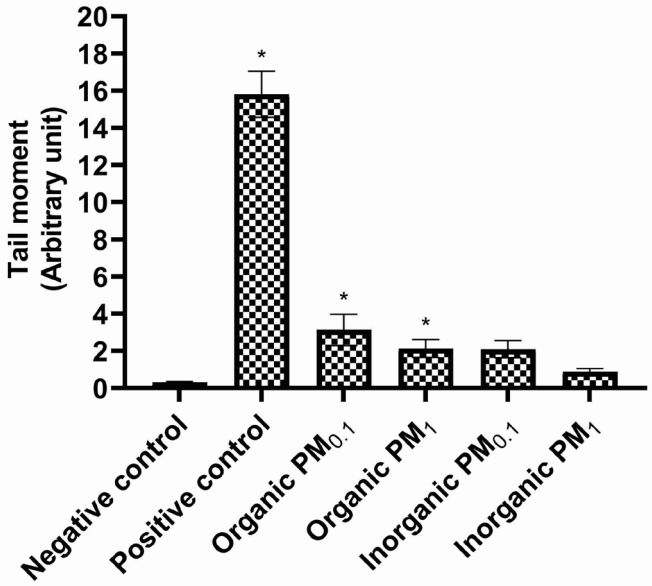

Fig. 2 : Tail moment (arbitrary unit) score based on the alkaline comet assay induced by different sizes of air samples. Results were expressed as means ± SEM of three independent experiments. *Significant difference ($p<0.05$) compared to the negative control.

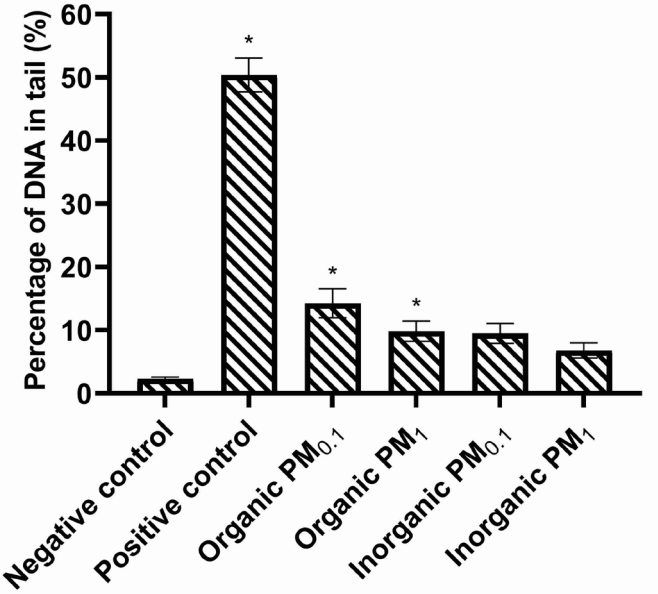

Fig. 3 : Percentage of DNA in tail (%) based on the alkaline comet assay induced by different sizes of air samples. Results were expressed as means ± SEM of three independent experiments. *Significant difference ($p<0.05$) compared to the negative control.

Table I : Level of DNA damage based on the alkaline comet assay

Treatment	Tail moment (Arbitrary unit) (Mean ± SEM)	DNA tail (%) (Mean ± SEM)
Negative control	0.31 ± 0.04	2.28 ± 0.30
Positive control (Hydrogen peroxide)	15.80 ± 1.24	50.40 ± 2.69
Organic $PM_{0.1}$ (IC_{25})	3.13 ± 0.85	14.24 ± 2.29
Organic PM_1 (IC_{25})	2.13 ± 0.48*	9.85 ± 1.60
Inorganic $PM_{0.1}$ (IC_{25})	2.09 ± 0.46#	9.50 ± 1.57
Inorganic PM_1 (IC_{25})	0.89 ± 0.17* #	6.80 ± 1.21

Note: Results were expressed as means ± SEM of three independent experiments. *significant different ($p<0.05$) between organic and inorganic particles, #significant different ($p<0.05$) between size $PM_{0.1}$ and PM_1.

percentage of DNA in the tail as well, as shown in Table I and Fig. 3. However, only cells treated with organic $PM_{0.1}$ ultrafine particles and PM_1 fine particles have a significantly higher tail moment and percentage of DNA in the tail as compared to the negative control. Generally, the organic and inorganic $PM_{0.1}$ ultrafine particles caused more DNA damage than their respective PM_1 fine particles counterparts. However, only inorganic $PM_{0.1}$ ultrafine particles (2.09 ± 0.46 a.u) caused significantly ($p<0.05$) higher tail moment scores as compared to inorganic PM_1 fine particles (0.89 ± 0.17 a.u.) in V79 cells. Meanwhile, it was also noted that the tail moment score in V79 cells treated with organic PM_1 fine particles (2.13 ± 0.48 a.u.) was significantly ($p<0.05$) higher as compared to inorganic PM_1 fine particles (0.89 ± 0.17 a.u.).

DISCUSSION

In this present study, we investigated the cytotoxicity and genotoxicity of fine (PM_1) and ultrafine ($PM_{0.1}$) particles using the V79 Chinese hamster lung cells. Our current findings showed that PM_1 had higher cytotoxic effects than $PM_{0.1}$ for both organic and inorganic extract. Although the smaller $PM_{0.1}$ particles may have the capability to penetrate into the cells easier, however current findings suggest that the cytotoxic effects of PM did not increase with decreasing aerodynamic diameters (16). In agreement with our current findings, Akhtar et al. reported that coarse and fine particles were more cytotoxic than the ultrafine particles (17). In the contrary, a previous study reported that ultrafine particles showed significantly higher cytotoxicity than all other PM with larger aerodynamic diameters (18). Hence, we suggest that the contradicting findings on the cytotoxic effects could be attributed to the variation in the chemical composition present in both the organic and inorganic extract of the PM_1 and $PM_{0.1}$.

Previously, we have reported that the PM_1 have higher organic carbon and elemental carbon than the $PM_{0.1}$ (11). The polycyclic aromatic hydrocarbons (PAH), which is one of the common compounds that can be found in the organic extract of PM, where found to be higher in PM_1 than in $PM_{0.1}$ (9). PAH is a class of organic compounds that can be formed from a series of anthropogenic activities, including biomass burning, traffic emission, diesel engines, and oil combustion, and has been demonstrated to possess cytotoxic, genotoxic, mutagenic and carcinogenic effects (19,20). Hence, we postulated that the higher cytotoxic effects of PM_1 organic extract could be explained by its higher levels of carbonaceous matter and PAH than $PM_{0.1}$. However, we cannot deny that the cytotoxicity of PM_1 could also be attributed to the other unidentified organic compounds that are present in the organic extract. Meanwhile, inorganic extract of PM_1 was also reported to have higher water-soluble inorganic ions (WSII) than the inorganic extract of $PM_{0.1}$, in particularly the NH_4^+ and SO_4^{2-} (11). Previously, the metal complexes of NH_4^+ and SO_4^{2-} have been reported to induce significant cytotoxic effects in cells (21,22). Additionally, although we do not study the metal contents of the PM extracts, the cytotoxic effects of PM could be also attributed to the present of transition metals as certain transition metals may induce oxidative damage and cause cellular damage (23).

In this present study, only cells treated with organic extract of PM_1 and $PM_{0.1}$ showed significant DNA damage, but not the inorganic extract. The genotoxic effects of the organic extract could be correlated to the presence of compounds such as PAH. PAH molecules may cause DNA damage to the cells once they undergo metabolic activation (24). PAH may lead to the formation of DNA adducts, thus interfering with the DNA repair machinery. If DNA damage is not repaired properly, it can lead to mutations and may cause cancer formation (19). For example, benzo(a)pyrene (BaP), is one of the examples of a PAH metabolite that is known to

cause cancer and recognized as a first-class carcinogen by the International Agency for Research on Cancer. Meanwhile, the inorganic extracts comprise predominantly sulphates and nitrates formed from sulphur dioxide and oxides of nitrogen. Although toxicological research in humans or animals does not suggest a prominent role for sulphate and nitrate in PM toxicity, epidemiological studies do (25).

Despite being less cytotoxic, our current findings demonstrated that $PM_{0.1}$ were more genotoxic than PM_1 for both organic and inorganic extract. Smaller PM fractions or particle sizes are suggested to be more harmful (4). Ultrafine particles have a higher surface area per unit mass along with surface reactivity and number, which some studies have found is essential in inducing DNA damage because more concentrations of toxic components, such as active redox chemical compounds adsorbed on the surface of PM to be delivered to target organs, especially lung cells (3). While cells are generally able to ingest most sizes of PM, those with a size less than 0.1 can traverse alveolar epithelial cells through transcellular diffusion across the lipid bilayer of the cell walls (4). It is also reported that some ultrafine particles can reach places of the body that larger particles cannot, thus increasing bio availability (26).

Although our current findings demonstrated that PM_1 and $PM_{0.1}$ possess genotoxic effects, however, the mechanism underlying the genotoxic effects of PM is still not fully understood. Previous studies have reported that the adverse health effects of PM stem from oxidative stress, which occurs due to the production of reactive oxygen species (ROS) and the activation of inflammatory cells in the affected cells (26,27). Previous studies have shown oxidative stress is the main factor in the occurrence of DNA damage by fine particle size (28,29). Furthermore, PM itself contains ROS (30). Other studies suggested that the absorbed PAH in PM can promote the production of ROS, which may subsequently lead to inflammation and DNA damage (31,32).

CONCLUSION

In conclusion, both organic and inorganic extracts of PM_1 and $PM_{0.1}$ were cytotoxic towards the V79 Chinese hamster lung cells, with PM_1 showing higher cytotoxicity as compared to $PM_{0.1}$. However, only the organic extracts, but not the inorganic extract of PM_1 and $PM_{0.1}$ showed significant genotoxic effects on V79 Chinese hamster lung cells. Moreover, this study also demonstrated that the ultrafine $PM_{0.1}$ was more genotoxic as compared to the PM_1. Further study is warranted to further investigate the chemical constituents that are responsible specifically for the genotoxicity of the organic and inorganic extracts from the PM_1 and $PM_{0.1}$ of the urban air sampled at the UKM Kuala Lumpur, Malaysia.

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