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IDENTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) IN THE AIR IN CUCUTA, COLOMBIA: GENOTOXIC EFFECT

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ABSTRACT

In recent years, there has been a boom in third-world countries, including those in Latin America, regarding the study of human exposure to combustion emissions, including ultrafine particles ($PM_{2.5}$) and mutagenic and carcinogenic components, such as aromatic hydrocarbons (PAHs). This study identified a group of polycyclic aromatic hydrocarbons (PAHs) recognized for their effects on health of the population in the city of Cucuta, Colombia; the PAHs found were: methylchrysene, benz[a]anthracene, benzo[j]fluoranthene, benzo[b] fluoranthene, benzo[a]pyrene, dibenzo[a,l]pyrene, dibenzo[a,e]pyrene, benzo[c]fluorene, benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene, and benzo[g,h,i]perylene. The genotoxic effect of the particulate matter, extracted with acetone and dichloromethane, was also evaluated in vitro using comet assay.

KEYWORDS: $PM_{2.5}$; PAHs; Benzo[a]pyrene; Gas chromatography; Comet assay.

IDENTIFICACIÓN DE HIDROCARBUROS AROMÁTICOS POLICÍCLICOS (HAPS) EN EL AIRE DE CÚCUTA-COLOMBIA: EFECTO GEN TÓXICO

RESUMEN

En los últimos tiempos ha tomado un gran auge en los países del tercer mundo, incluidos los de América Latina, el estudio de la exposición humana a las emisiones de combustión, incluyendo las partículas ultrafinas ($PM_{2.5}$) y componentes mutagénicos y carcinógenos como los compuestos aromáticos policíclicos (HAPs). En este trabajo se identificó en la ciudad de Cúcuta, un grupo de hidrocarburos aromáticos policíclicos (HAPs), reconocidos por su afectación a la salud de la población; los HAPs encontrados fueron: Metilcriseno, Benzo[a]antraceno, Benzo[j]fluoranteno, Benzo[b] fluoranteno, Benzo[a] Pireno, Dibenzo [a,l] pireno, Dibenzo[a,e] pireno, Benzo[c] fluoreno, Benzo[k] fluoranteno, Indeno[1,2,3-cd] pireno, Dibenzo[a,h]antraceno y Benzo[g,h.i]perileno. Adicionalmente también se evaluó *in vitro* usando el ensayo cometa, el efecto genotóxico del material particulado $PM_{2.5}$ extraído con Acetona y Diclorometano.

PALABRAS CLAVE: $PM_{2.5}$; hidrocarburos aromáticos policíclicos; benzo(a)pireno; cromatografía de gases; ensayo cometa.

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IDENTIFICAÇÃO DE HIDROCARBONETO DE POLICÍCLICOS AROMÁTICOS (HAPS) NO AR DE CÚCUTA- COLÔMBIA: EFEITO GEN TÓXICO

RESUMO

Nos últimos tempos, o estudo de exposição humana às emissões de combustão, incluindo as partículas ultrafinas ($PM_{2.5}$) e componente mutagênicos e carcinógeno como os compostos aromáticos policíclicos (HAPs) tiveram nos países do terceiro mundo um crescimento incluindo na América Latina. Neste trabalho identificou-se na cidade de Cúcuta, um grupo de hidrocarboneto de policíclicos aromáticos (HAPs), reconhecidos pela sua afetação à saúde da população; os HAPs encontrados foram: Metilcriseno, Benzo[a]antraceno, Benzo[j]fluoranteno, Benzo[b] fluoranteno, Benzo[a] Pireno, Dibenzo [a,l] pireno, Dibenzo[a,e] pireno, Benzo[c] fluoreno, Benzo[k] fluoranteno, Indeno[1,2,3-cd] pireno, Dibenzo[a,h]antraceno y Benzo[g,h.i]perileno. Mais também avaliou-se in vitro usando o teste cometa, o efeito genotoxico do material particulado $PM_{2.5}$ extraído com Acetona e Diclorometano.

PALAVRAS-CHAVE: $PM_{2.5}$; Hidrocarboneto de Policíclicos Aromáticos; Benzo(a)pireno; Cromatografia de gases; Teste cometa.

1. INTRODUCTION

Atmosphere contaminants are substances that can affect humans, animals, vegetation, and materials. Airborne toxins are contaminants known to cause or suspected of causing negative health effects including cancer and immune, development, or neurological effects (USEPA, 2004). Several of these airborne contaminants differ in their physical-chemical characteristics and can be grouped into four categories: gaseous contaminants (ozone, SO_2, NOx, CO, volatile organic compounds), persistent organic contaminants, heavy metals, and particulate matter (PM_{10} and $PM_{2.5}$) (fine and ultrafine). The contaminants can affect the respiratory system and cause pulmonary and systemic issues including inflammation, tissue reconstruction, and carcinogenesis (Dagouassat & Boczkowski, 2012).

Breathable fraction particles (PM_{10} and $PM_{2.5}$) can penetrate and be deposited in the tracheobronchial and alveolar regions or the respiratory tract (Vinitketkumnuen et al., 2002). In terms of its effects on human health, the breathable fraction of particulate matter (PM) is the greatest concern since long-term exposure to PM has been associated with a greater incidence of pulmonary and cardiovascular illnesses and cancer (Pope et al., 2002; Brunekreef & Holgate., 2002).

Breathable particles are a mix of airborne contaminants that contain various metals such as Pb, Cd, or Cr, many of which are toxic (Shah, et al., 2006). In urban areas, heavy metals come from industrial activity and vehicular emissions (Wu et al., 2007; Zheng et al., 2010).

$PM_{2.5}$ contains mutagenic PAHs and carcinogens that can cause oxidative damage to DNA and lead to cardiovascular and reproductive issues (Lewtas, 2007). The increase in morbimortality, especially due to cardiovascular and pulmonary illnesses, is associated with the increase in fine atmospheric particles and the damages they cause through oxidative stress (Zhang & Cui, 2001). Oxidative stress is associated with abnormal levels of reactive oxygen species (ROS), which are thought to be related to carcinogenic processes (Milaeva, 2011). Oxidative damage to DNA is very important in carcinogenesis. 8-Oxo-7, 8-dihydroguanine (8-oxoGua) is an abundant mutagenic lesion split by oxoguanine DNA glycosylase 1 (OGG1); 8-OxoGua excretions have been associated with exposure to air pollution (Loft et al., 2012). Environmental exposure to PM has been associated with increased risk of lung cancer through alterations to methylation processes. (Hou et al., 2011). The mutagenicity and carcinogenicity of urban air have been associated with combustion emissions (Claxton & Woodall, 2007). The products of incomplete combustion contain gaseous carcinogens, such as benzene, aldehydes, alkenes (1,3-butadiene, for example) and PAHs such as pyrene (Claeys et al., 2004).

Studies on the distribution of ultrafine particle ($PM_{2.5}$) mass sources show that diesel and gasoline

vehicles are one of the main sources of PM emissions (Maykut et al., 2003; Watson & Chow, 2001; Schauer et al., 1996; U.S. EPA, 2004). Diesel characterization studies show that its particles contain relatively large quantities of organic mutagenic compounds (Lewtas et al., 1979). 3-nitrobenzanthrone (3-NBA) has been isolated from diesel and air particles and has been shown to be a powerful direct action mutagen (Enya et al., 1997) that can also form in atmospheric reactions (Arlt, 2005). In humans, inhalation is the most frequent access route for PAHs into the organisms. This is why the respiratory tract and the lungs are generally involved in the damaging agent's translocation process through the blood and tissues (Hałatek et al., 2005).

A cancer epidemiology study on occupational exposure to diesel exhaust emissions provided the greatest evidence of carcinogenicity in humans (U.S. EPA, 2002). The most common types of cancer associated with diesel exhaust gases are: lung, bladder, and lymphatic tissue (Bhatia, Lopipero & Smith, 1998; Lipsett & Campleman, 1999). According to the EPA, diesel combustion constitutes one of the main sources of carcinogens for human beings, which reach the body through inhalation (U.S. EPA, 2002). Studies have been completed to evaluate the population exposed to PAHs using environmental monitoring information (Touma, Cox & Tikvart, 2006; McCarthy, et al., 2009).

In addition, the U.S. EPA identified the most common PAHs and classified them as "the 16 priority PAHs" (State of California EPA.(2008).: naftalene, acenaphthylene, acenaphthene, fluorenone, anthracene, phenanthrene, fluoranthene, pyrene, benzo[a] anthracene, chrysene, benzo[a]pyrene, benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[ghi]perylene, dibenz[a,h]anthracene, and indeno[1,2,3-c,d]pyrene. A PAH subgroup has been identified by the International Cancer Research Agency (IARC for its initials in Spanish) as carcinogens for animals and has been studied by the U.S. EPA as potential carcinogens for humans. These compounds must be routinely monitored in environmental studies on organic toxins and they are: benzo[a]anthracene (2B), chrysene (2A), benzo[a] pyrene (1), benzo[b]fluoranthene (2B), benzo[j]fluoranthene (2B), benzo[k]fluoranthene (2B), dibenz[a,h] anthracene (2A), and indeno[1,2,3-c,d]pyrene (2B).

There is no information regarding genotoxicity levels or the presence of PAHs in the air in the northeastern region of Colombia and the border region with Venezuela (Cucuta). In this study, ultrafine particles ($PM_{2.5}$) and volatile organic compounds were collected during the period between July and December of 2011 with a low-volume device near a road in Cucuta that is heavily traveled by vehicles that run on diesel and gasoline. The PAHs present in Cucuta's $PM_{2.5}$ were extracted with two solvents independently of each other (acetone and dichloromethane) and were analyzed with gas chromatography using an FID. The genotoxicity associated with the Cucuta-Colombia air was evaluated in vitro using comet assay.

2. METHODOLOGY

2.1. Sampling

The $PM_{2.5}$ monitoring was completed with a Partisol-Plus 2025 from Ruprecht & Pataschnick with a flow of 16.6 L/min according to OMS and EPA guidelines.

2.2. Sampling Site

The $PM_{2.5}$ breathable fraction was monitored in Cucuta in the northern region of Santander located in the eastern mountain chain of Colombia with geographical coordinates of 72° 25 West longitude and 7°20 North latitude, at an altitude of 300masl and an atmospheric pressure of 650 mmHg. The device was installed on the roof of the Universidad de Pamplona building located on Diagonal Santander. The environmental samples were obtained in 24-hour samplings for a total of 24m^3 air volume sampled every three days. The samples were taken during the period between July and December of 2011.

The $PM_{2.5}$ sample site has particular characteristics given that it is located in a mixed sector (residential and commercial) and next to a road with a high flow of diesel and gasoline combustion vehicles.

2.3. Means of Collection

47mm diameter Teflon filters with a pore size of 2 microns were used. They were subjected to a

temperature of 200°C for 24h. After cooling, they were placed in a desiccator, and once completely cool, they were weighed and then adapted in the Partisol filter holder. After the monitoring process, the filters located in the used filter holders were stored in the laboratory at -20°C until the extractions were completed.

2.4. Ultrasound Extraction of Organic Matter from $PM_{2.5}$ Filters

The PAHs present in the organic fraction of the $PM_{2.5}$ filters were extracted using ultrasound in an ultrasonic bath (Branson 1510, model 1510R-MT). Dichloromethane (200ml) and acetone (200ml) were used separately as extraction solvents. The $PM_{2.5}$ filters were initially placed in a beaker with 20ml of solvent for an extraction period of 15 minutes at a temperature of 23°C-24°C. The extract was collected, and 20ml were added again to complete the extraction for another 15 minutes. This process was repeated until all 200ml of the solvent were used.

2.5. Concentration of Organic Matter

Once the global extract was obtained (200 ml), it was concentrated in a Heidolph rotary vacuum evaporator model Laborota 400-1 at the solvent's boiling temperature at 150rpm until approximately 10ml remained. The extract was then transferred to two 5ml vials. The global extract was used to determine the PAHs through FID gas chromatography and for the comet assay. The vial for the comet assay was dried and dissolved in DMSO.

The PAH samples were dried with Na_2SO_4 in order to eliminate residual water and prepare the simple for chromatographic analysis. They were stored in amber jars and kept refrigerated at 4°C.

2.6. Polycyclic Aromatic Hydrocarbon (PAH) Identification

To identify the PAHs present in the $PM_{2.5}$ of the Cucuta air extracted with dichloromethane and acetone, an Agilent Technologies 6890A Plus Series II Hewlett-Packard Plus gas chromatography device with an FID (flame ionization detector) was used. The column used was Agilent DB-EUPAH 20m longitude, 0.18mm diameter, 0.25μm internal diameter (5% Phenyl Methylpolysiloxane). For PAH identification, the Restek 16 hydrocarbon pattern was used (catalog #31841 EPA Method 8310 PAH Mixture). The qualitative identification of the PAHs present in the global extract was completed according to the following conditions: FID at 320°C mix (mL/min): air 400 – H_2 30 –N_2 45. 1μl was injected in splitless mode at 320°C. The initial temperature was 45°C during 0.8 min, and it was increased as follows: 45°C/min until 200°C, 2.50°C/min until 225°C, 3°C/min until 266°C, 5°C/min until 300°C, 10°C/min until 320°C for 4.50min. The analysis time per sample was 41.21 minutes. The carrier gas was helium with a flow of 20mL/min. The injector temperature was 250°C.

DNA Damage Detection. Comet Assay. Comet assay is a highly sensitive technique for evaluating the damage and reparation of DNA in any type of eukaryote cell. In its alkaline version, comet assay allows for detection of simple strand breaks and areas sensitive to alkali that originate during repair, which causes the formation of comet tails (Ayala Mayte, 2004). In general, the test's basic principle is the migration of DNA in an agarose matrix under electrophoresis conditions. Then, when the cell is observed through a microscope, it shows a comet-like appearance with a head (nucleus region) and tail (formed by nuclear fragments that have migrated toward the anode), which is the source of the comet assay's name, alluding to the DNA migration pattern caused in damaged cells.

Treatment. After separating the lymphocytes from the whole blood, 200μl were taken, and the treatment or control were added. The following doses were analyzed: 50μg, 100μg, and 200μg. For the positive control, H_2O_2 25mM was used, and for the negative control, DMSO 12%. They were then incubated for 1h at 37°C. 75μl of low melting point (LMA) agarose were taken and mixed with 10μl of treated cells. The previous mixture was then added to the basal lamina, the cover was immediately put on, and the mixture was taken to incubation for 6 minutes at 4°C. The cover was then removed, and another 75μl of LMA agarose were added. The mixture was incubated for 6 minutes at 4°C. After the 6 minutes were completed, the cover

was removed and the mixture was incubated for 1h at 4°C in a lysis solution. The plates were then washed with PBS and placed in an electrophoresis chamber for 30 minutes without plugging the chamber in. The chamber was then plugged in for 30 minutes at 300 milliamperes. Once the time period was completed, the plates were removed from the chamber and washed with neutralizing solution. They were allowed to dry, 30μl of ethidium bromide were immediately added, and the covers were put on for cell reading. Olympus U-RFKT50 fluorescence was then observed through the microscope with the 25x objective, and the DNA migration of 200 cells was measured. The measurements were made using the program CometScore.

To determine genotoxin damage, intervals were established depending on the damage to the negative control, which in this case was of 41μm. Therefore, cells that showed damage between 0 and 41μm were classified as cells with damage type 0. **Table 1** shows the different intervals and damage types.

Statistical Analysis. The variance homogeneity was determined using the Levene test. If the data's behavior was parametric, a variance analysis (ANOVA) was applied. If the data were not parametric, Mann-Whitney and Wilcoxon tests were used. The Dunnett test was used to determine the level of significance between the treatment and the control, as well as the Tukey test for multiple comparisons. The values were expressed as the mean ± standard deviation (X ± SD), and the tests were considered significant with a $p \leq 0.05$.

2.7. Polycyclic Aromatic Hydrocarbon (PAH) Identification with Gas Chromatography/FID

To have a reference of possible PAHs present in the air in the city of Cucuta, a pattern containing 16 pure polycyclic aromatic hydrocarbons was first injected.

3. RESULTS AND DISCUSSION

In the $PM_{2.5}$ of the air in Cucuta, the following heavy metals have been found in the study reports of Gutierrez, Quijano & Quijano P. (2012): Cd, Cr, Pb, Zn, and Fe.

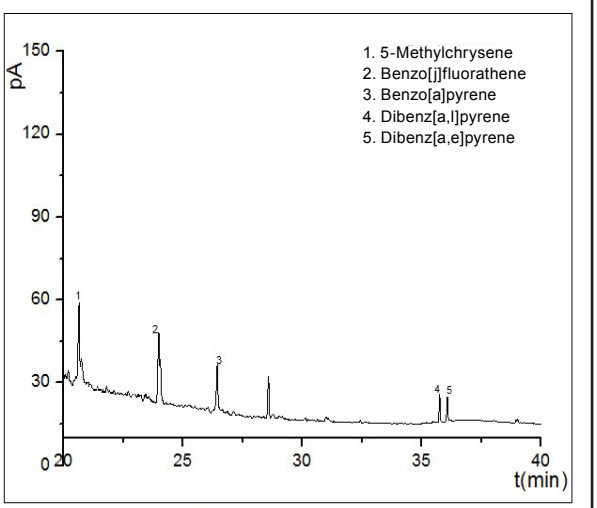
Table 1. Intervals and classes of damage for analyzed cells

Interval in µm	Type of Damage	Class of Damage
0 – 41	0	No damage
42 – 83	1	Low damage
84 – 125	2	Medium damage
>126	3	High damage

These metals come from mobile sources that use diesel and gasoline in combustion, which corroborates the observation of Fukino et al. (1982) which attributed the mutagens present in the air to mobile sources.

Figure 1 shows the chromatographic profile of the global extract for organic compounds present in the $PM_{2.5}$ in the city of Cucuta using dichloromethane as an extraction solvent.

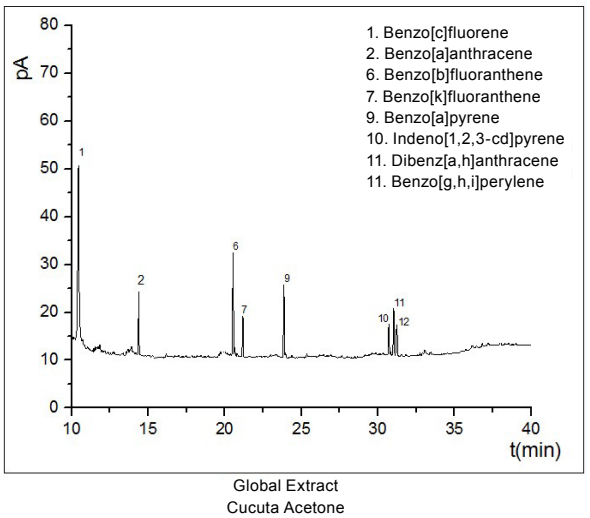
Figure 1. Chromatogram of the organic matter in the $PM_{2.5}$ of the Cucuta air extracted with dichloromethane.



As can be observed in this chromatograph, the PAHs found in the $PM_{2.5}$ samples of the air in Cucuta extracted with dichloromethane are: 5-methylchrysene, benzo[j]fluoranthene (2B), benzo[a]pyrene (1), dibenz[a,l]pyrene, and dibenz[a,e]pyrene.

Figure 2 shows the chromatographic profile of the PAHs present in the $PM_{2.5}$ from the city of Cucuta extracted with acetone.

Figure 2. Polycyclic aromatic hydrocarbons present in the air in Cucuta extracted with acetone.



As can be observed in this chromatogram, the PAHs found in the $PM_{2.5}$ samples of the air in Cucuta extracted with acetone are: benzo[c]fluorene, benzo[a] anthracene (2B), benzo[b]fluoranthene (2B), benzo[k] fluoranthene (2B), benzo[a]pyrene (1), indeno[1,2,3-cd]pyrene (2B), dibenzo[a,h]anthracene (2A), and benzo[g,h.i]perylene (3).

When DCM and acetone are compared as extraction solvents for the organic matter in the $PM_{2.5}$ (PAHs), we can conclude that acetone is the better solvent since it identifies other PAHs not extracted by DCM.

The damage caused by $PM_{2.5}$ extracts in the city of Cucuta was analyzed using comet assay for a total of 200 cells through treatment in three independent experiments. The indicator of genotoxic damage in each cell is the breakage of its DNA, which manifests itself as a comet such that the greater the damage, the longer the tail of the comet. To show that the damage to DNA in cells is due to treatment and not to factors that lead to cell death, cellular viability was determined after treatment, and it was found that viability always remained above 95% (**Tables 2** and **3**). Damage categories were also established to show that the damage was not uniformly distributed in all cells, which could be an advantage due to the fact that exposure did not necessarily affect all cells, thereby diminishing the risk. As can be observed in **Tables 2** and **3**, the cells that showed spontaneous damage did not exceed a tail length of 43μm, and the majority of cells (80%) are in the 0 interval, showing that the majority of cells did not have damage or show very little damage. Only 8% of the calls showed type 1 damage, and none of the cells showed type 2 or 3 damage. Similarly, with the 50μg dose of $PM_{2.5}$, we can observe that 37% of the cells had type 0 damage, 53% had type 1 damage, 10% had type 2 damage, and 1% had type 3 damage. As can be observed in **Tables 2** and **3**, as we increased the concentration of the extract, the type of damage also increased until it reached a high level of damage, as in the case of the 150μg dose, in which 65% of the cells showed a high level of damage, 11% showed a medium level of damage, and 4% showed a low level of damage. We can also observe in **Table 3** that the doses that caused the greatest frequency of DNA damage in cells also showed greater lengths of DNA migration (tails). This could indicate that the genotoxins that cause the most damage to DNA also affect a greater number of cells. It is worth highlighting the fact that when the genotoxicity of the $PM_{2.5}$ extracted with acetone was analyzed (**Table 3**), it showed an increase slightly higher than the genotoxicity found when the extraction was completed with dichloromethane (**Table 2**). These results could be attributed to the fact that when the extraction was completed with acetone, more compounds were obtained, and these could lead to greater genotoxicity.

As we can observe in **Tables 2** and **3**, all the doses of $PM_{2.5}$ analyzed caused damage in the DNA of human lymphocytes. This indicated that the majority of the mutagens that reach the population in air particles can penetrate to the nucleus of human cells and damage their DNA. These results concur with studies that have been completed in Colombia and other countries (U.S. EPA. 2002 ; Arlt, 2005; Meléndez, Martínez & Quijano, 2012). These findings indicate that part of the genotoxicity shown by the air in Cucuta is caused by the PAHs found given that there is sufficient evidence correlating the presence of these compounds with a risk to human health. For example, benzo[a]pyrene is classified in group 1 as a carcinogen for humans by the EPA (State of California EPA, 2008); dibenz[a,h]anthracene is classified in group (2A) as a probable carcinogen for humans; benzo[a]anthracene, benzo[b]fluoranthene, and

indeno[1,2,3-cd]pyrene are classified in group (2B) as possible carcinogens for humans; benzo[g,h,i]perylene is classified in group 3 as non-classifiable as a carcinogen for humans: the EPA and the International Cancer Research Agency (IARC, 2010), emphasize dibenz[a,h] pyrene as a possible carcinogen.

Considering that genotoxic damage was observed in human lymphocytes, we can conclude that:

The $PM_{2.5}$ breathable fraction in an area with heavy vehicular flow in Cucuta in the north of Santander could be one of the risk factors that contributes to the increase in the cancer rate among the exposed population due to the fact that it can cause mutations in the genome of exposed cells and can also penetrate into the nucleus of human lymphocytes and cause genotoxic damage to their DNA.

The air in Cucuta showed genotoxicity probably due to the following PAHs: benzo[a]pyrene, benzo[a] anthracene, benzo[b]fluranthene, benzo[k]fluranthene, indeno[1,2,3-cd]pyrene, and dibenz[a,h]anthracene, as well as the metals Cd, Cr, Pb, Zn, and Fe, which were found in the $PM_{2.5}$ organic matter at this location. This genotoxicity is related to the emissions of diesel motors that circulate in the city of Cucuta.

In the extraction of PAHs with acetone, benzo[g,h,i]perylene could be detected in the city of Cucuta. Benzo[g,h,i]perylene is an indicator of PAHs emitted by diesel and gasoline motor exhausts.

Table 2. Genotoxicity (expressed in tail length in µm) in human lymphocytes exposed to different doses of breathable fraction $PM_{2.5}$ extracted with dichloromethane in the city of Cúcuta.

Treatment	Average Damage ± SD	Type of Damage				% Damaged Cells	% Viability
		0	1	2	3		
DMSO 1 %	38 ± 8	135	15	0	0	11	98
H_2O_2 25mM	187 ± 15	5	15	50	80	97	95
50 µg	70 ± 9	88	53	9	0	41	96
100 µg	103 ± 13	15	19	100	16	90	97
150 µg	135 ± 15	7	10	22	109	95	98

All the values are derived from 150 pieces of information obtained in three independent experiments. The ranges for the types of damage were obtained in the following manner: from 0 to 41µm, type 0 damage = no damage; from 42 to 83µm, type 1 damage = low damage; from 84 to 125µm, type 2 damage = medium damage, and above 126µm, type 3 damage = high damage. The percentage of damaged cells and cellular viability after 1h of treatment were also determined.

Table 3. Genotoxicity (expressed in tail length in µm) in human lymphocytes exposed to different doses of breathable fraction $PM_{2.5}$ extracted with acetone in the city of Cúcuta.

Treatment	Average Damage ± SD	Type of Damage				% Damaged Cells	% Viability
		0	1	2	3		
DMSO 1 %	38 ± 8	135	15	0	0	11	98
H_2O_2 25mM	187 ± 15	5	15	50	80	97	95
50 µg	70 ± 9	88	53	9	0	41	96
100 µg	103 ± 13	15	19	100	16	90	97
150 µg	135 ± 15	7	10	22	109	95	98

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