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Genotoxicity and Cytotoxicity Induced In Vitro by Airborne Particulate Matter ($PM_{2.5}$) from an Open-Cast Coal Mining Area

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Abstract: This study evaluates the cytotoxic and genotoxic effects of $PM_{2.5}$ collected from an open-cast coal mining area in northern Colombia. Cyclohexane (CH), dichloromethane (DCM), and acetone (ACE) extracts were obtained using Soxhlet extraction to isolate compounds of different polarities. Human lymphocytes were exposed to the extracted compounds, and cytotoxicity and genotoxicity were assessed using the cytokinesis block micronucleus (CBMN) and comet assays, incorporating FPG and ENDO III enzymes to detect oxidative DNA damage. Chemical analysis revealed that the organic fractions consisted mainly of modified hydrocarbons and volatile organic compounds. The CBMN assay showed a significant increase in micronuclei in binucleated (MNBN) and mononucleated (MNMONO) cells and nucleoplasmic bridges (NPB) in exposed lymphocytes. The comet assay revealed substantial oxidative DNA damage, particularly with the ACE extract, which significantly increased oxidized purines and pyrimidines. DCM induced similar effects, while CH showed moderate effects. CREST immunostaining revealed aneugenic activity, particularly in cells exposed to ACE and DCM extracts. These results suggest that polar fractions of $PM_{2.5}$, likely containing metals and modified PAHs, contribute to DNA damage and chromosomal instability. The study highlights the need to monitor the composition of $PM_{2.5}$ in mining regions to implement stricter environmental policies to reduce exposure and health risks.

Keywords: genotoxicity; chromosomal instability; $PM_{2.5}$ extracts; oxidative DNA damage; polar fractions

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1. Introduction

$PM_{2.5}$, defined as particulate matter 2.5 microns or less in diameter, can penetrate deep into the lungs and enter the bloodstream, causing significant adverse health effects [1]. In mining regions, $PM_{2.5}$ is generated by blasting, drilling, transportation, and coal combustion. This fine particulate matter (PM) can remain suspended in the air, posing an important health risk to nearby communities [2,3]. Exposure to PM in proximity to open-cast coal mines has been associated with increased morbidity from respiratory diseases [4], cardiopulmonary conditions, and lung cancer [5], leading to its classification as a probable human carcinogen by the International Agency for Research on Cancer (IARC) [6].

In vitro studies have demonstrated that $PM_{2.5}$ exhibits both genotoxic and cytotoxic effects probably caused by its complex chemical composition. Multiple studies have established a strong correlation between DNA damage and fine particulate matter exposure [7,8]. Increasing evidence suggests that the genotoxic effects of PM are primarily due to its organic fraction [9,10], which may contain a mixture of carcinogenic compounds such as polycyclic aromatic hydrocarbons (PAHs). In coal mining areas, PAHs may be released due to incomplete combustion [11] when coal stored in surface mines can spontaneously combust when exposed to direct sunlight and high temperatures; these compounds can form DNA adducts that disrupt normal cellular processes [6], lead to genetic mutations and contribute to oxidative DNA damage by generating free radicals [12,13]. In addition, some studies suggest that chemical elements in the PM, either in the water-soluble or in the insoluble fraction [14], may catalyze reactions that exacerbate oxidative stress-induced DNA damage [14]. These elements generate reactive oxygen species (ROS), leading to oxidative damage to cellular components such as DNA, proteins, and lipids, which in turn results in both genotoxic (DNA damage, mutations, chromosomal instability) and cytotoxic (cell death) effects [9,15]. The cytotoxicity of $PM_{2.5}$ is manifested through cellular damage or cell death, including apoptosis and necrosis. Apoptosis is often triggered by mitochondrial dysfunction, which involves disrupting mitochondrial membrane potential and releasing pro-apoptotic factors like cytochrome c. This pathway is closely linked to oxidative stress and DNA damage, highlighting the interconnected nature of $PM_{2.5}$'s genotoxic and cytotoxic effects [16–18]. Complex airborne particle mixtures released in open-cast coal mines contain high concentrations of chemical elements, including lead (Pb), cadmium (Cd), arsenic (As), chromium (Cr), nickel (Ni), manganese (Mn), titanium (Ti), and zinc (Zn) [19]. These elements, contained mainly in the $PM_{2.5}$ fraction, can be absorbed by the body through inhalation [20].

Despite growing evidence of the adverse health effects of $PM_{2.5}$, most existing studies have focused on urban environments, and there is a significant gap in understanding genotoxic and cytotoxic effects, particularly in open pit mines mining regions where PM concentrations are often elevated. Additionally, in open-cast mining, toxic substances contained in the $PM_{2.5}$ fraction form complex mixtures [2,21] that interact synergistically [22], amplifying their genotoxic potential. Ambient air quality standards have been implemented as a public health measure to mitigate these risks. However, these standards may not adequately address the chemical composition of $PM_{2.5}$, which can pose significant health risks even at concentrations below the established regulatory thresholds [16]. Some authors argue that the most critical aspect of atmospheric PM that has yet to be fully addressed in ambient air standards is the chemical composition of $PM_{2.5}$ [23–25].

The coal-rich region of northern Colombia is one of the major areas for surface coal mining with most reserves concentrated in La Guajira peninsula, producing approximately of 32 Mtons/year. Colombian environmental regulations establish specific limits for PM_{10} and $PM_{2.5}$ concentrations with permissible 24 h exposure limits of 75 $\mu g/m^3$ for PM_{10} and 37 $\mu g/m^3$ for $PM_{2.5}$ [26]. The current national air quality policy aims to implement stricter targets by 2030, reducing these limits to 30 $\mu g/m^3$ for PM_{10} and 15 $\mu g/m^3$ for $PM_{2.5}$. However, the chemical characterization of PM is not routinely performed in Colombia [27].

This study aims to address this gap by evaluating the genotoxic and cytotoxic effects of $PM_{2.5}$ collected from an open-pit coal mining region in northern Colombia on human

lymphocytes. $PM_{2.5}$ extracts of different polarities were analyzed to determine the relative toxicity. The primary objective was to assess chromosomal instability using the cytokinesis-block micronucleus cytome (CBMN-cyt) assay. In addition, oxidative DNA damage was quantified using the modified comet assay with FPG and ENDO III enzymes to assess the oxidative stress induced by these particles. CREST staining was used to distinguish centromere-positive from centromere-negative micronuclei (MNs) and differentiate between aneugenic and clastogenic effects. This approach provides a detailed assessment of the genotoxic and cytotoxic effects of $PM_{2.5}$ exposure and contributes to understanding the associated health risks in coal mining regions.

2. Materials and Methods

2.1. Location of Sampling Sites and Study Area

The study area is situated in the foothills of the Serranía del Perijá, which is a semi-arid region in the southern portion of the La Guajira department in northeastern Colombia. This region is located between the municipalities of Barrancas, Albania and Hatonuevo [28], is significantly affected by coal mining activities and is home to Wayúu indigenous settlements and several rural communities [29]. The sampling sites for $PM_{2.5}$ were situated within the direct influence zone of mining operations and near the Puerto Bolívar coal terminal. Based on our previous studies on the effects of mining activities on DNA damage parameters in residentially exposed populations [19,30], five particulate sampling areas were defined in the municipalities of Barrancas, Hatonuevo, and Puerto Bolívar. In Barrancas, three locations were established: the Wayúu communities of Provincial (72°44′10.0″ W; 11°01′24.0″ N) and San Francisco (72°45′58.0″ W; 11°00′08.0″ N) and the Afro-Colombian community of Chancleta (72°40′36.0″ W; 11°03′08.0″ N). As a result of the elevated air pollution levels, the latter community was eventually relocated [31]. In the municipality of Hatonuevo, samples were collected from the Wayúu community of Cerro de Hatonuevo (72°45′40.0″ W; 10°55′38.0″ N); in Puerto Bolívar, the Wayúu community of Media Luna (71°59′49.0″ W; 12°13′33.0″), located near the Puerto Bolívar coal terminal, was selected for sampling. Finally, a reference area was selected for the Manaure community in Mayapo (72°46′60.0″ W; 11°39′00.0″ N). This area is geographically and environmentally distant from the mining operations and serves as a control area to account for baseline levels of PM and any potential background environmental pollution unrelated to coal mining. This reference area allows a more apparent distinction between mining-related pollutants and naturally occurring or regional environmental factors (Figure 1).

2.2. Collection of $PM_{2.5}$ Samples

For $PM_{2.5}$ collection, a total of 30 atmospheric aerosol samples were collected for 24 h between August and December 2015, starting at 9:00 a.m. Considering that the collection was conducted in a semi-arid region, with minimal seasonal variations, the data obtained are representative of the prevailing conditions throughout the rest of the year. A BGI PQ200 FRM sampler, equipped with a $PM_{2.5}$ inlet and WINS impactor (as per CFR40 part 50, appendix L), was used with an airflow rate of 16.7 L/min. The sampler was calibrated with a MesaLabs DryCal Definer 220 flow calibrator (Brandt Instruments, Prairieville, LA, USA). On-site calibrations involved verifying the volumetric flow by comparing the calibrated orifice results with volumetric flow controller tables. The samples were collected using 46.2 mm diameter polytetrafluoroethylene (PTFE) filters, supported with polymethyl pentene (PMP) rings (Tisch Environmental Inc., Cincinnati, OH, USA). These hydrophobic, chemically inert filters have low background interference, making them ideal for gravimetric and chemical analysis of particulate matter. With a pore size of 2.0 µm, the filters effectively trapped $PM_{2.5}$ with minimal flow resistance. Filters were equilibrated in a desiccator under controlled conditions (T = 26.5 ± 1 °C, RH = 30.4 ± 5%) and weighed until consistent values were obtained before and after sampling. The PM samples were tagged, preserved, and transported at 4 °C for additional analysis. The

filters were measured using a digital microbalance (Radwag MYA11-3Y) with a precision of 10^{-6} g.

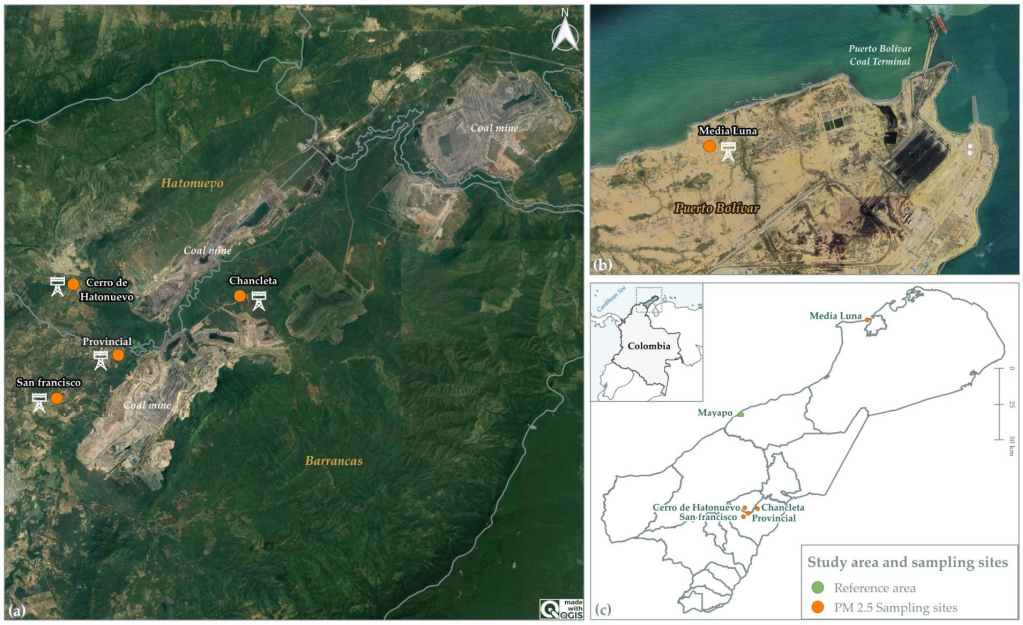


Figure 1. Study area and sampling sites. (**a**) Open pit coal-mining areas, (**b**) Puerto Bolívar coal terminal, (**c**) general location of the study area.

2.2.1. Chemicals

General cell culture reagents were purchased from (Sigma-Aldrich, St. Louis, MO, USA). Acetone (ACE) was purchased from (Merck K45815214434, Kenilworth, NJ, USA), cyclohexane (CH) from Panreac 131250.0314, Barcelona, Spain), and dichloromethane (DCM) from (Honeywell 10624214, Seelze, Germany). The solvent stock solutions were dissolved in dimethyl sulfoxide (DMS) (Carlo Erba–445103, Chicago, IL, USA) and stored at −20 °C in sealed, sterile glass vials. For the treatments, each stock was dissolved in fetal bovine serum and phenol red-free RPMI 1640 medium (Sigma R-8755, USA).

2.2.2. Extractable Organic Matter (EOM) Determination

Filters from the five sampling sites directly impacted by coal mining activities and from the control area were sequentially extracted using three different solvents. Three quarters of the PTFE filters from each site were extracted together in a Soxhlet apparatus, following the protocol outlined in USEPA Method 3540C (1996). The extraction process for the filters at each site lasted 24 h with each solvent applied in a series of 8 h cycles at a temperature of 59 °C. The solvents were used in the following order: 60 mL of CH from Merck, followed by 60 mL of DCM from Honeywell, and finally 60 mL of ACE from Merck. After extraction, the solutions for each solvent (CH, DCM, and ACE) at each site were concentrated to 1.5 mL using a rotary vacuum evaporator (Heidolph Instruments, Schwabach, Germany). Preliminary analysis of the extractable organic matter (EOM) from the extracts revealed a similar chemical composition across the sampled coal mining areas (see Figure S1). As a result, we pooled the extracts by solvent type to create a representative profile, producing a combined extract for each solvent from the mining-affected sites. This approach ensures a comprehensive overview of the organic fractions across the mining-impacted area, maintaining consistency while reflecting the overall impact. The extract from the reference area was analyzed to distinguish contaminants associated with mining activities from naturally occurring or regional environmental factors. The concentration of extractable organic matter (EOM) in $\mu g/m^3$ was calculated by dividing the total EOM collected per filter by the total volume of air sampled. The EOM concentration in percentage

represents the proportion of EOM in the $PM_{2.5}$ sample. The concentration of EOM in % represents the percentage of EOM in the $PM_{2.5}$.

2.2.3. Cleanup

The concentrated extracts were separated using a 20 cm × 1.5 cm column packed with pre-cleaned silica gel (heated for 20 h at 110 °C). The column was initially eluted with 20 mL of hexane: dichloromethane mixture (9:1, *v*:*v*), followed by 30 mL of hexane: dichloromethane (4:1, *v*:*v*), and finally 10 mL of dichloromethane: methanol (9:1, *v*:*v*). The extracts were then concentrated using a rotary evaporator, transferred to vials, and stored at −20 °C until further analysis.

Subsequent analysis of the samples was conducted using high-resolution gas chromatography-mass spectrometry (GC–MS).

2.2.4. High-Resolution Gas Chromatography-Mass Spectrometry (GC–MS)

The GC-MS analysis was conducted using a Shimadzu GC2010 gas chromatograph coupled to a GCMS QP2010 mass spectrometer. The separation was achieved with an Optima 5 MS column, measuring 30 m in length, 0.25 mm in internal diameter, and 0.25 µm in film thickness. The column oven temperature was initially set at 40.0 °C with a temperature ramp up to 260.0 °C at a rate of 10.0 °C/min. The injection temperature was 250.0 °C, and the injection was performed in splitless mode with a sampling time of 1.00 min. The pressure was maintained at 49.5 kPa with a total flow of 4.0 mL/min, column flow of 1.0 mL/min, and purge flow of 3.0 mL/min. The linear velocity was 36.1 cm/sec, and helium was used as the carrier gas in a constant flow mode.

The mass spectrometer was operated with an ion source temperature of 260.0 °C and an interface temperature of 260.0 °C. The solvent cut time was set at 3.00 min. The detector gain mode was set to relative with a gain of 0.00 kV and a threshold of 1000. The mass spectrometric analysis was performed in scan mode with a scanning speed of 666 amu/sec and a mass range from 40.00 to 350.00 m/z. The event time was 0.50 s with the scan starting at 3.00 min and ending at 42.00 min.

The obtained chromatograms in. qgd format files were processed in GCMSsolution software (Shimadzu Corp., Tokyo, Japan) for peak integration to correct any overlapping peaks or inadequate peak separations. The mass spectral search algorithm, integrated with GCMSsolution, was employed to identify the compounds in question. The peaks corresponding to the experimentally obtained mass spectra were then compared with the NIST-05 database. A similarity threshold of 80% was employed to ascertain the reliability of compound identification based on the retention indexes, coded RetIndex, based on the Linear Temperature Programmed Retention Index (LTPRI) for the Shimadzu GC2010.

2.3. Blood Samples and Lymphocyte Isolation

Lymphocytes were obtained from blood samples collected from three healthy volunteers, non-smokers, without recent exposure to X-rays or coal mining residues, ages 30–35 years old. A survey was conducted to collect data on exposure to other factors, such as the use of psychoactive drugs, recent severe viral diseases, and exposure to other pollutants. The participants were previously informed about the study's purpose. After informed consent was obtained, blood samples were collected aseptically by venipuncture. Approximately 14 mL of peripheral blood from each donor was obtained by venipuncture in tubes with EDTA (Becton-Dickenson, NJ, USA)) for subsequent lymphocyte extraction. The lymphocytes were isolated by centrifugation using the Ficoll–Histopaque method and washed with sterile 1×PBS and RPMI 1640 medium without phenol red (Sigma R8755, USA). The initial viability of the mononuclear cells was determined by the trypan blue exclusion method, considering a viability criterion of 90% per patient [2].

Primary Human Lymphocyte Cultures and Assessment of Cytotoxicity

Approximately 9×10^4 cells per well were cultured in 96-well plates using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2% phytohemagglutinin (PHA), 1% penicillin–streptomycin solution, and 1% L-glutamine. The cells were incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. All primary cultures from donors were processed simultaneously to ensure reproducibility.

After the initial 24 h incubation period, 20 µL of serial dilutions of the evaluated extracts (ACE, CH, DCM) was added, resulting in final concentrations in the culture medium of 0.001, 0.020, 0.045, 0.090, 0.180, 0.360, 0.730, 1.470, 2.940, and 5.88 mM. Two exposure periods were employed to assess cytotoxicity: 4 h for acute cytotoxicity and 72 h for chronic cytotoxicity. ACE, CH, and DCM served as blanks, H_2O_2 (hydrogen peroxide) was used as a positive control, and untreated cells were used as a negative control.

To prevent cross-contamination due to volatilization between wells, a sterile sheet of Alumna Seal™ (RPI Corp., Mt. Prospect, IL, USA) was placed over the plate before covering it. Following the incubation periods, cell viability was assessed using the XTT assay, which relies on the reduction of the yellow tetrazolium salt XTT to formazan by dehydrogenase enzymes in metabolically active cells, resulting in an orange water-soluble product [28,32]. After each treatment, the wells were washed, and 200 µL of deionized water was added. Absorbance was measured at 595 nm using a BioRad (Hercules, CA, USA) microplate reader. Data were corrected using the blanks with the mean blank-corrected absorbance value of the negative control set to 100% viability. The absorbance for each treatment group well was then expressed as a percentage relative to this concurrent negative control. Results were presented as dose–response curves, and the IC50 values for each extract were calculated using nonlinear regression based on the Hill slope ($R^2 > 0.95$) in GraphPad Prism version 10.

2.4. High-Throughput Single-Cell Gel Electrophoresis and Modified Comet Assay

The comet assay is another fast, sensitive, and effective method for assessing genetic damage [33].

The alkaline Comet assay was conducted in accordance with the protocol delineated by Espitia-Peréz et al. [2], incorporating several alterations to establish a high-throughput variant proficient in processing numerous samples [34].

After 24 h of primary culture, lymphocytes were washed twice with phosphate buffered saline (PBS) to remove FBS. The ACE, CH and DCM concentration ranges for the SCGE assay did not exceed 30% of acute cytotoxicity [35,36]. H2O2 was used as the positive control, and RPMI 1640 medium without FBS was used as the negative control. All treatment conditions and experimental designs were performed following [37]. After treatment, cells were centrifuged and washed 3 times with PBS. An aliquot of the cell suspension was analyzed for acute cytotoxicity with the trypan blue dye exclusion assay. The remaining cell suspension (10 mL) was mixed with 90 mL of 0.5% low melting point agarose (Sigma) in PBS without Ca^{2+} and Mg^{2+}.

Additionally, an alkaline comet assay with lesion-specific enzymes (ENDO III and FPG) was used to detect oxidized pyrimidines and purines, respectively [38]. Following lysis, minigels were washed with enzyme buffer and incubated with ENDO III (1:1000 for 30 min) or FPG (1:1000 for 45 min) at 37 °C alongside control gels treated with buffer alone. DNA unwinding, electrophoresis, and staining were conducted as described for the high-throughput comet assay [2]. The contribution of 8-oxoG and oxidized pyrimidines to DNA damage was calculated by subtracting the % Tail DNA values for enzyme-treated gels from those of the buffer-only controls. Significant differences of 7–11% in % Tail DNA were observed for enzyme-treated samples compared to controls.

2.5. Cytokinesis-Block Micronucleus (CBMN) Assay

The CBMN-cyt assay includes key biomarkers such as the frequency of micronuclei in binucleated cells (MNBNs), which indicates chromosome breakage and/or complete chromosome loss; micronuclei in mononucleated cells (MNMONOs), representing chromosome damage induced and expressed in vivo prior to culture; nucleoplasmic bridges (NPBs), markers of DNA misrepair and/or telomere end fusions; and nuclear buds (NBUDs), which are linked to the removal of amplified DNA and/or DNA repair complexes [39]. The CBMN assay was conducted following the methodology previously described by Fenech in 2007 [39] and Pastor et al. in 2023 [40]. Briefly, after 24 h of primary culture, cells were treated with each experimental concentration of ACE, CH, and DCM for 4 h at 37 °C, 5% CO_2 in air. Methyl methane sulfonate (MMS) 1 mM was used as the positive control. Cell cultures in complete RPMI 1640 medium were the negative controls. Lymphocytes cultures were established in 4.5 mL of RPMI 1640 medium (Sigma R8758, USA) supplemented with 2 mM l-glutamine (Sigma A5955, USA), 10% fetal bovine serum (Gibco 15000-044, Brazil), 100 µL/mL antibiotic–antimycotic (Sigma A5955, USA) and 2% phytohemagglutinin (Sigma L8754, USA).

Cultures were kept in a 37 °C environment without light for 44 h with 5% CO_2. After 44 h, 6 µg/mL of cytochalasin B (Sigma, C6762) was introduced. Following the incubation, lymphocytes were collected through centrifugation, treated with a methanol/acetic acid solution for fixation, and stained with Diff-Quick. Microscopy was used to analyze two thousand binucleated cells for micronuclei (MNs), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs). The frequency of micronuclei in 1000 mononucleated (MONO) cells was also examined. The cytochalasin B proliferation index (CBPI) was determined based on cell ratios. A blind analysis was conducted, and three independent experiments were carried out per treatment [41].

CREST Immunostaining

The CBMN-cyt assay in combination with CREST antibody distinguishes MN containing one or several whole chromosomes (CREST+), which are positively labeled (centromere positive MN, due to aneugenic effect), or acentric chromosome fragments, which are unlabeled (CREST−) due to the absence of centromeres (centromere-negative MN due to clastogenic effect).

After 24 h of primary culture, cells were treated with each experimental concentration of ACE, CH and DCM for 4 h at 37 °C in a humidified atmosphere containing 5% of CO_2. Cells were transferred to a frosted slide and air-dried for 5 min. Then, cells were fixed with methanol and acetic acid pre-chilled at −20 °C for 10 min and air-dried again for 10 min. The CREST immunostaining technique was performed according to the protocol described by [19].

2.6. Statistical Analysis

The statistical analyses were conducted using the R software, version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria), with the functions from the base stats package. All experiments were independently repeated three times. A pooled-data analysis from all donors was performed for each assay. Mean and median comparisons were performed for CBMN-cyt parameters and % Tail DNA in the Comet assay. Dunnett's test was conducted to determine if there was a significant difference in DNA damage compared to the negative control. To determine the ACE, CH, and DCM influence over CBMN-cyt parameters, we conducted a Kruskal–Wallis test, since the frequencies were not normally distributed. Additionally, we used the Mann–Whitney U non-parametric test to compare the induced CBMN-cyt parameters frequency to the negative control. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Chemical Characteristics of ACE, CH and DCM Extracts

Three solvents were chosen for their specific abilities to extract a wide range of pollutants from $PM_{2.5}$ samples, covering both non-polar and polar fractions. The selected solvents were CH, DCM, and ACE. CH was selected for its effectiveness in extracting non-polar compounds, including aliphatic hydrocarbons, PAHs, and certain non-polar oxidized hydrocarbons. As a result, it represents an optimal solvent for isolating hydrophobic substances commonly found in PM. DCM was selected as the second solvent due to its capability to dissolve a broad spectrum of organic compounds, particularly those with medium and low polarity. Its extraction efficiency is notably superior to benzene, with approximately 26% higher efficacy, indicating its suitability for complex environmental samples. ACE, the third solvent, was expected to serve a dual function: the extraction of both organic compounds and polar or semi-polar inorganic materials, including nitrates, sulfates, and trace metals, as well as water-soluble organic compounds. This multi-solvent approach ensures a comprehensive chemical characterization of the $PM_{2.5}$ samples, encompassing a diverse range of pollutant types [42].

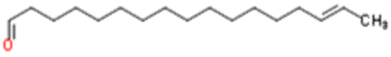
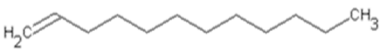
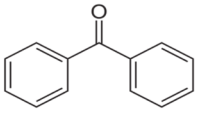
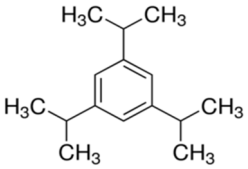
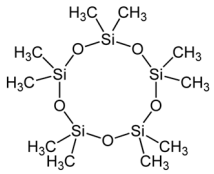
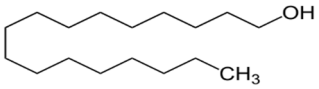
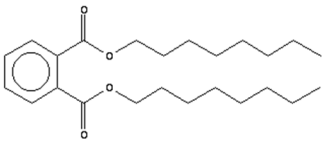
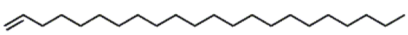
Notably, the highest EOM concentrations were observed in Provincial, while the lowest concentrations were recorded in Mayapo (Figure S1A). A preliminary analysis of EOM revealed a consistent pattern of organic compounds and an increased percentage of CH-soluble material across mining-affected locations, indicating the predominance of non-polar compounds (Figure S1B). Comprehensive chemical characterization was subsequently performed on extracted materials. Table 1 presents the principal chemical composition of the extracts obtained using ACE, CH, and DCM in coal mining areas. The chemical analysis unexpectedly revealed the absence of PAHs commonly associated with coal mining activities. The lack of PAHs specifically linked to coal mining activities suggests a difference from the anticipated chemical profile. Instead, the analysis showed that most of the detected compounds corresponded to modified hydrocarbons and volatile organic compounds with alkenes being the most dominant. These findings indicate that other hydrocarbon derivatives may be more common than PAHs in the region's $PM_{2.5}$.

Among the compounds analyzed in the ACE extracts, several alkenes were identified, including 1-hexadecene ($C_{16}H_{32}$), E-15-heptadecenal ($C_{17}H_{32}O$), and 1-nonene (C_9H_{18}). The predominant compounds found were 1-hexadecene ($C_{16}H_{32}$), 3-tridecene (Z) ($C_{13}H_{26}$), 1-dodecene ($C_{12}H_{24}$), and 1-octadecene ($C_{18}H_{36}$). In addition, benzophenone ($C_{13}H_{10}O$), a polar oxygenated hydrocarbon (Oxy-PAH) commonly associated with $PM_{2.5}$, was detected. These results suggest that the ACE solvent was particularly effective at extracting volatile and oxygenated compounds, which may be linked to spontaneous combustion processes or industrial activities in the area [43,44].

The DCM extracts revealed the presence of high molecular weight compounds, including 2,4,6-triisopropylbenzene ($C_{15}H_{24}$), which is recognized as a marker of fossil fuel combustion from energy production, heating, transportation, or industrial applications [45–47]. Additionally, compounds such as cyclopentasiloxane ($C_{16}H_{48}O_{10}Si_9$) and 1-heptadecanol ($C_{17}H_{36}O$) were detected, indicating the presence of organic materials that may originate from industrial processes or lubrication activities [48].

The CH extract demonstrated the presence of non-polar hydrocarbons, including 1-tricosene ($C_{23}H_{46}$), 1-docosene ($C_{22}H_{44}O$), and phthalic acid esters such as di-n-octyl phthalate ($C_{24}H_{38}O_4$)[49–51]. Phthalates, or phthalic acid esters (PAEs), are pervasive contaminants in diverse environmental compartments and have been linked to adverse health effects due to their capacity to bioaccumulate and their potential to cause reproductive harm [52,53].

Table 1. Main chemical constituents of ACE, CH and DCM extracts in coal mining areas determined by GC/MS.

Extract	Formula	Name	CAS Number	Chemical Structure
ACE	$C_{16}H_{32}$	1-Hexadecene	629-73-2	
	$C_{17}H_{32}O$	E-15-Heptadecenal	700381-35-7	
	C_9H_{18}	1-Nonene	124-11-8	
	$C_{13}H_{26}$	3—Tridecene (Z)	41446-53-1	
	$C_{12}H_{24}$	1-Dodecene	112-41-4	
	$C_{13}H_{10}O$	Benzophenone	119-61-9	
	$C_{18}H_{36}$	1-Octadecene	112-88-9	
DCM	$C_{15}H_{24}$	2,4,6-triisopropylbenzene	717-74-8	
	$C_{16}H_{48}O_{10}Si_9$	Cyclopentasiloxane	145344-72-5	
	$C_{17}H_{36}O$	1-Heptadecanol	1454-85-9	
CH	$C_{23}H_{46}$	1-Tricosene	18835-32-0	
	$C_{24}H_{38}O_4$	Di-n-octyl phthalate	117-84-0	
	$C_{22}H_{44}O$	1-Docosene	1599-67-3	

3.2. Cytotoxicity of ACE, CH and DCM Extracts in Human Lymphocyte Cultures

As shown in Figure 2, the ACE, CH, and DCM extracts caused a dose-dependent decrease in the viability of human lymphocytes. The ACE extract exhibited the strongest cytotoxic effect, with a mean inhibitory concentration (IC50) of 0.17 mM, as determined by nonlinear regression analysis, indicating it is the most potent at inducing cell death. In contrast, the CH extract showed the lowest cytotoxicity, with an IC50 of 0.47 mM, suggesting a higher concentration is required to achieve the same inhibitory effect as ACE. The DCM extract demonstrated intermediate cytotoxicity, with an IC50 of 0.26 mM, being less toxic than ACE but more potent than CH.

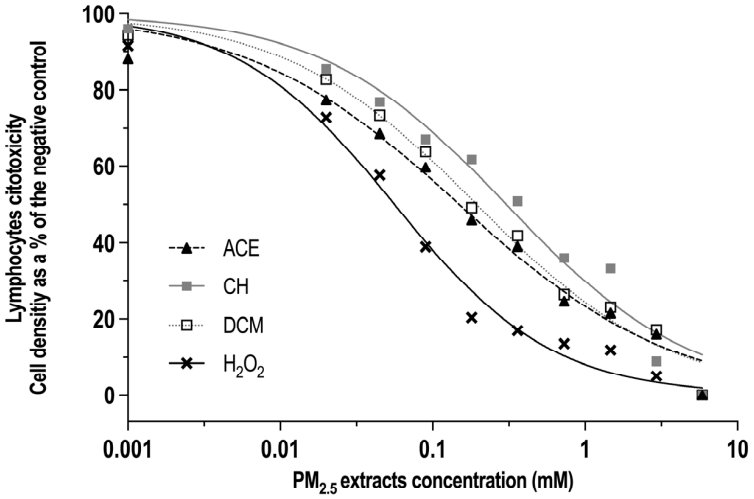


Figure 2. A log-linear plot of the lymphocyte cell cytotoxicity of ACE, CH and DCM extracts. The concentration was considered cytotoxic when cell survival was 50%. The results are shown as the mean of three independent experiments.

3.3. Genotoxicity and Oxidative Damage Induced by ACE, CH and DCM Extracts in Human Lymphocyte Cultures

The concentration–response curves for genomic DNA damage and acute cytotoxicity induced by ACE, CH, and DCM extracts, as well as H_2O_2, are shown in Figure 3. All extracts caused a dose-dependent increase in DNA damage, which was measured by the DNA percentage in the tail (% Tail DNA). The ACE and DCM extracts showed slightly higher % Tail DNA values compared to the CH extract.

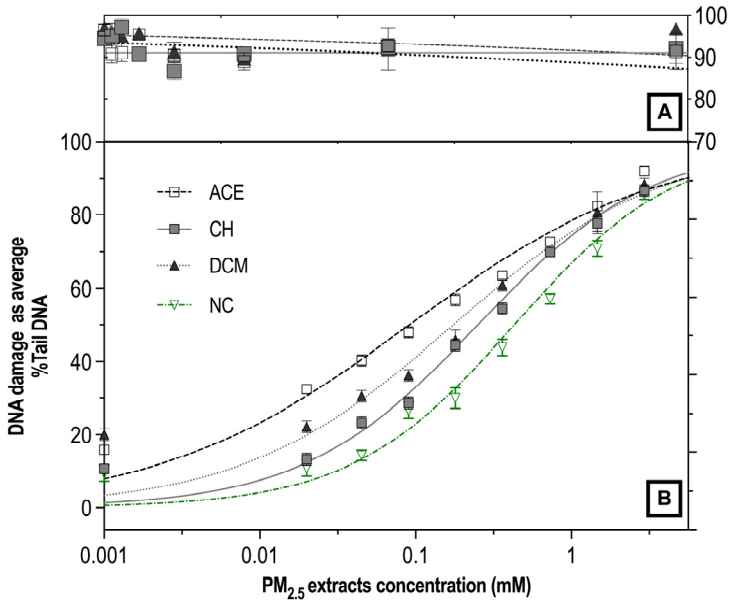


Figure 3. SCGE analysis of ACE, CH and DCM extracts in lymphocytes. (**A**) Acute toxicity of lymphocytes from treatment groups used in the SCGE assay. (**B**) Log-linear plot of the genotoxic concentration–response curves. H_2O_2 was used as the positive control.

A significant increase in DNA damage was observed at all concentrations of ACE and DCM extracts compared to the negative control ($p < 0.05$). However, this increase in DNA damage was only significant for the CH extract at the highest concentrations (0.47 and 0.94 mM). Benzene derivatives and oxy-PAHs were more prevalent in the ACE and DCM extracts. Previous research has suggested that organic extracts with moderate

and polar polarity may also contain substantial amounts of inorganic materials, such as nitrates, trace metals, and organic compounds. These results suggest that more polar extracts, like ACE and DCM, are more likely to induce DNA damage than less polar extracts. This may be attributed to their higher content of reactive compounds, which increases their interaction with genetic material and other cellular components, leading to a more pronounced toxic effect.

Figure 4 shows the results of oxidative damage induction using the modified comet assay with FPG and ENDO III endonucleases. The ACE extract caused a significant increase in oxidized purines and pyrimidines in exposed lymphocytes compared to the negative control. Similar effects were observed with the DCM extract; however, the oxidative damage induced by DCM and CH was comparable particularly with ENDO III treatment. Additionally, while FPG and ENDO III treatment revealed a slight increase in oxidized purines compared to oxidized pyrimidines in lymphocytes treated with the CH extract, this difference was not statistically significant.

3.4. Effect of ACE, CH and DCM Extracts on CBMN-Cyt Assay Parameters and CREST Staining

The effects of ACE, CH, and DCM extracts on various CBMN-Cyt assay parameters, including MNBN, MNMONO, NBUD, NPB, and nuclear division index (NDI), were evaluated in human lymphocytes (Table 2). As extract concentrations increased, so did the frequency of MNBN for all three extracts with the strongest effects observed at higher concentrations. For the ACE extract, a concentration of 0.34 mM resulted in a significant increase in MNBN (7.53 ± 8.25, $p < 0.05$) and MNMONO (0.96 ± 1.95, $p < 0.05$) compared to the negative control. Similarly, for the CH extract, a concentration of 0.94 mM showed a significant increase in MNBN (6.59 ± 4.63, $p < 0.05$) and MNMONO (0.76 ± 1.69, $p < 0.05$). The DCM extract at 0.52 mM also resulted in significant increases in MNBN (7.15 ± 7.67, $p < 0.05$), MNMONO (0.68 ± 1.28, $p < 0.05$), and NPB (0.40 ± 1.02, $p < 0.05$) compared to the negative control. These results were consistent with the positive control (MMS at 1 mM), which showed significant increases across all categories. Additionally, as the extract concentrations increased, the NDI decreased, indicating both cytotoxic and genotoxic effects in the exposed lymphocytes.

The analysis of CREST+ and CREST- micronuclei in human lymphocytes exposed to varying concentrations of ACE, CH, and DCM extracts revealed aneuploidy-inducing activities at specific concentrations of ACE and DCM. In lymphocytes treated with the ACE extract, a dose-dependent increase in CREST+ micronuclei were observed, reaching its peak at 0.360 µM, indicating significant aneugenic activity. Similarly, the DCM extract showed a marked increase in CREST+ micronuclei at the same concentration (0.360 µM), suggesting a strong potential to induce aneuploidy. In contrast, the CH extract showed only moderate aneugenic activity, with slight increases in CREST+ micronuclei at 0.009 µM and 0.180 µM, and no significant increase at 0.360 µM compared to the negative control. These findings highlight the greater capacity of the ACE and DCM extracts to induce chromosomal alterations in human lymphocytes compared to the CH extract (Figure 5).

Table 2. CBMN-Cyt parameters frequencies after ACE, CH and DCM exposure in lymphocytes.

Extract	Concentrations (mM)	MNBN	MNMONO	NBUD	NPB	NDI
ACE	0	2.52 ± 2.12	0.05 ± 0.24	0.80 ± 1.60	0.10 ± 0.31	2.13 ± 0.14
	0.05	4.34 ± 1.67	0.86 ± 0.09	0.55 ± 0.33	0.28 ± 0.62	2.33 ± 0.11
	0.08	4.45 ± 3.67	0.98 ± 0.16	0.35 ± 0.78	0.31 ± 0.21	2.05 ± 0.08
	0.17	6.30 ± 6.23	0.33 ± 0.71	0.43 ± 0.94	0.53 ± 1.54	1.63 ± 0.28
	0.34	7.53 ± 8.25 *	0.96 ± 1.95 *	0.68 ± 1.11	0.56 ± 1.98	1.61 ± 0.45
CH	0	1.41 ± 0.28	0.15 ± 0.36	0.43 ± 0.14	0.17 ± 0.89	2.16 ± 0.11
	0.05	2.13 ± 0.84	0.19 ± 0.61	0.51 ± 0.40	0.19 ± 0.51	2.0 ± 0.31
	0.23	3.16 ± 0.52	0.18 ± 0.59	0.48 ± 0.95	0.22 ± 0.54	1.68 ± 0.18
	0.47	4.28 ± 2.56	0.21 ± 0.61	0.60 ± 1.06	0.33 ± 0.66	1.62 ± 0.28
	0.94	6.59 ± 4.63 *	0.76 ± 1.69 *	0.83 ± 0.38	0.65 ± 0.17	1.52 ± 0.15

Table 2. *Cont.*

Extract	Concentrations (mM)	MNBN	MNMONO	NBUD	NPB	NDI
DCM	0	2.08 ± 0.38	0.50 ± 0.40	0.61 ± 0.17	0.20 ± 0.56	2.0 ± 0.09
	0.05	2.68 ± 0.30	0.49 ± 0.23	0.55 ± 0.65	0.33 ± 0.62	1.87 ± 0.13
	0.13	4.75 ± 3.72	0.47 ± 1.07	0.67 ± 0.78	0.09 ± 0.30	1.96 ± 0.78
	0.26	5.54 ± 3.98	0.55 ± 1.65	0.70 ± 0.13	0.95 ± 0.22	1.70 ± 0.19
	0.52	7.15 ± 7.67 *	0.68 ± 1.28 *	0.90 ± 0.35	0.40 ± 1.02 *	1.69 ± 0.20
Positive control MMS	1	8.15 ± 1.67 *	1.15 ± 0.96 *	1.35 ± 1.78 *	1.23 ± 0.80 *	1.30 ± 0.11*

* Significant difference compared with the negative control group within a column. $p < 0.05$.

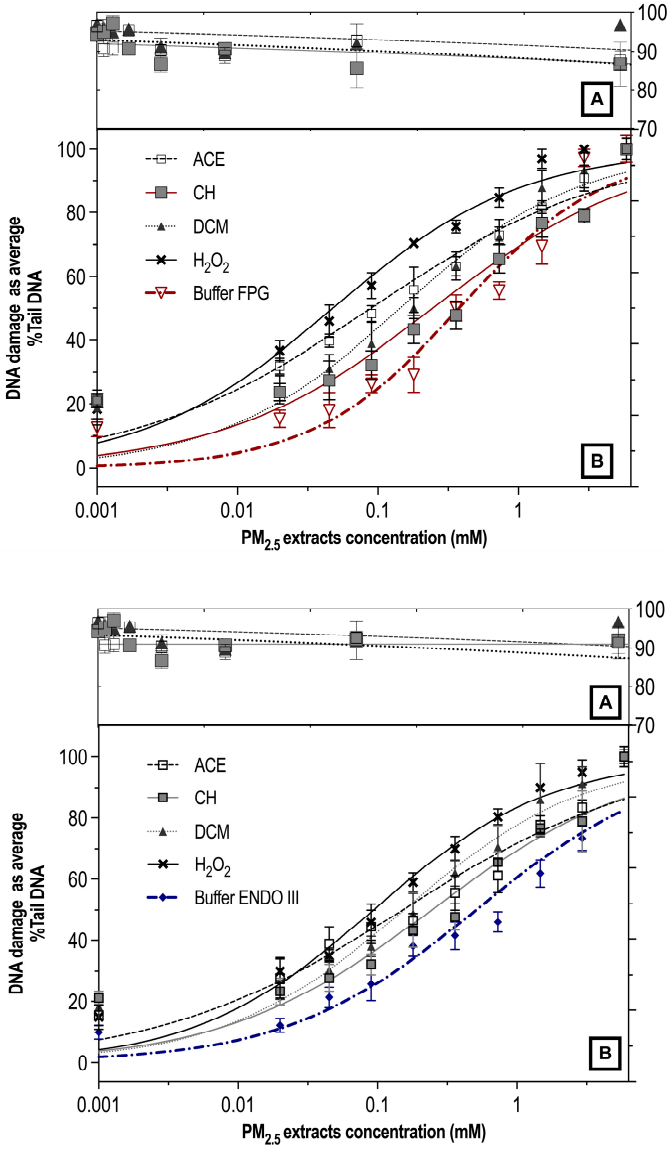


Figure 4. Analysis of the oxidative damage caused by ACE, CH and DCM extracts exposure in lymphocytes after treatment with ENDOIII and FPG enzymes in the modified SCGE. Right (Upper panel) (**A**) Acute toxicity of lymphocytes from treatment groups used in the SCGE assay. Right (Lower panel) (**B**) Log-linear plot of the genotoxic concentration–response curves. H_2O_2 was used as the positive control.

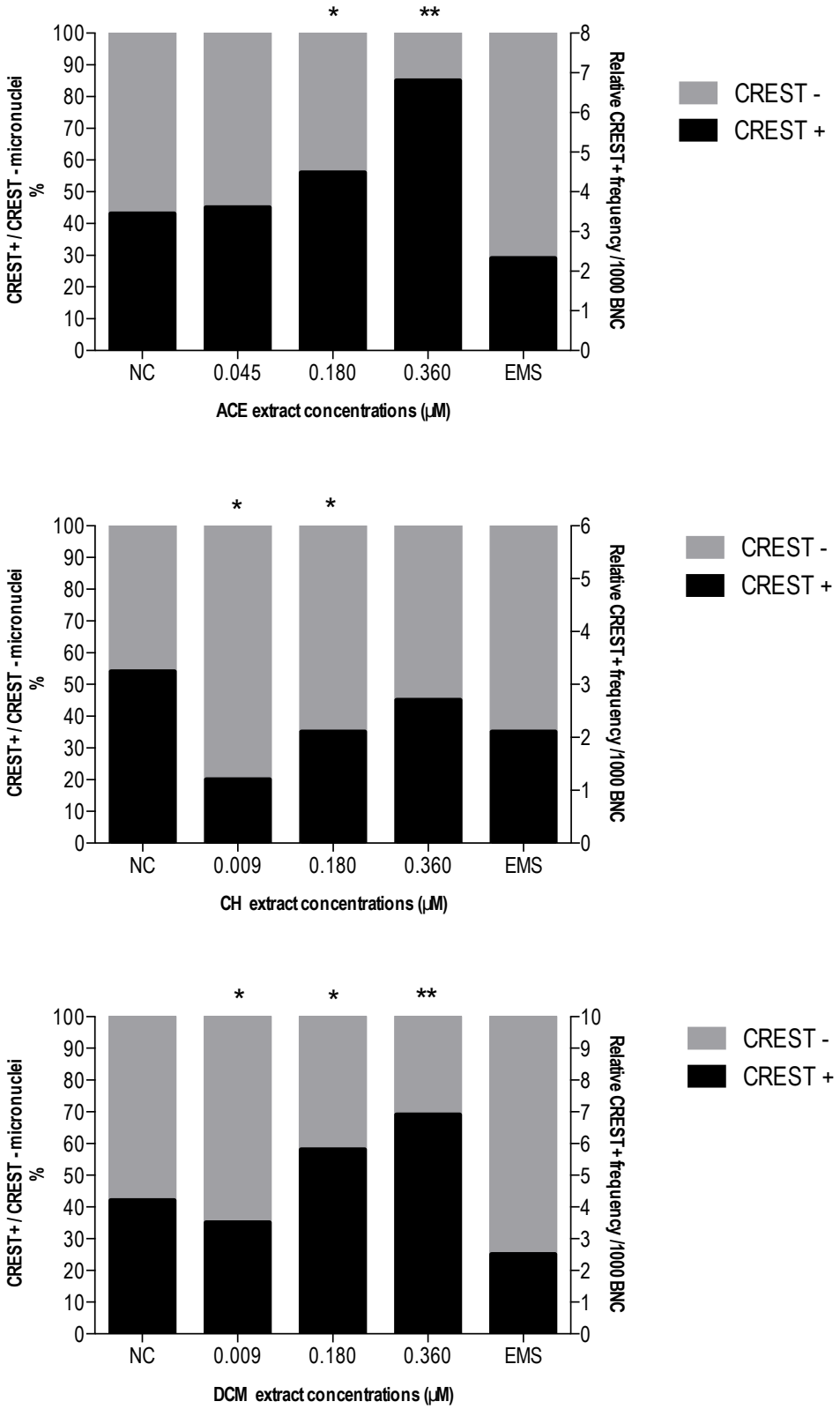


Figure 5. Percentages of CREST+/CREST- micronuclei induced by exposure to ACE, CH and DCM extracts. * Significant difference in relation to the negative control; $p \leq 0.05$; ** Significant difference in relation to the negative control; $p \leq 0.01$.

4. Discussion

Our results suggest that the chemical composition of the ACE, CH, and DCM extracts reflects the specific environmental conditions of the coal mining area. Contrary to initial expectations, the absence of PAHs indicates that coal mining activities may not be a direct source of PAHs in this environment. Instead, the presence of modified hydrocarbons and alkenes, particularly in the ACE extracts, points to ongoing combustion processes, possibly linked to spontaneous coal combustion, as suggested in the literature [53].

The detection of Oxy-PAHs in the ACE extracts, associated with $PM_{2.5}$, highlights the potential health risks posed by these compounds, which are known to be more toxic than their parent PAHs [54,55]. This finding aligns with studies that indicate Oxy-PAHs formation through primary fuel combustion and subsequent reactions with atmospheric pollutants such as ozone (O_3) and nitrogen oxides (NOx) [56]. The environmental conditions in La Guajira—marked by high temperatures and intense solar radiation—likely accelerate the formation of Oxy-PAHs, as demonstrated by other studies [57,58].

Furthermore, the detection of phthalates in the CH extracts raises additional concerns due to their widespread presence in the environment and their potential to cause DNA damage and disrupt reproductive hormone levels [59–61]. These findings underscore the complexity of pollutant profiles in coal mining areas and highlight the need for comprehensive monitoring and regulation, particularly with regard to the composition of $PM_{2.5}$, to mitigate potential health risks.

The results of the cytotoxicity assays indicate that the ACE extract demonstrates the highest cytotoxic potential among the three solvents tested, which is followed by DCM and CH. This finding is consistent with previous studies that have proposed a direct correlation between the polarity of the extracting solvents and their cytotoxic effects [62,63]. Highly polar extracts, such as those obtained with ACE, tend to contain nitroaromatics, aromatic amines, and aromatic ketones. These compounds are formed in the atmosphere when organic substances, even those that are non-mutagenic, are exposed to NOx and sunlight [64]. The increased cytotoxicity observed with more polar solvents like ACE and DCM may be attributed to their enhanced ability to extract compounds that amplify the uptake and retention of fine particles within the respiratory system, potentially leading to more severe adverse effects [63]. This highlights the importance of considering solvent polarity in toxicological studies, as it significantly influences the types of compounds extracted and their subsequent biological effects. The findings emphasize the need for further research into the mechanisms driving these effects and the implications for human health, particularly in regions with high levels of atmospheric pollution.

All extracts—ACE, CH, and DCM—induced genotoxic effects in human lymphocyte cultures, as evidenced by the increased % Tail DNA. The higher genotoxicity observed with ACE and DCM extracts aligns with the detection of benzene derivatives and Oxy-PAHs, which are compounds known for their DNA-damaging properties [65–67]. These findings are supported by previous research showing that such compounds, particularly when associated with inorganic materials like nitrate and trace metals, can generate ROS, leading to oxidative stress and subsequent DNA damage [68,69].

The use of the Comet assay with FPG and ENDOIII endonucleases provided further insight into the nature of the oxidative damage, showing that the ACE extract induced a significant increase in oxidized purines and pyrimidines compared to the control. Although DCM and CH also induced oxidative damage, the effects were comparable and less pronounced than those of ACE, particularly in the ENDOIII treatment [70,71].

The connection between oxidative stress and carcinogenesis, highlighted by biomarkers such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), underscores the potential long-term health risks associated with exposure to these environmental contaminants [72]. The correlation between FPG-Comet assay changes and cellular ROS, as noted by Zhao et al. [73], further emphasizes the relevance of these findings in understanding the genotoxic and oxidative mechanisms at play. For biomonitoring purposes, the enzymatic detection of

DNA oxidized products via the comet assay offers a practical and informative approach to assessing environmental genotoxicity [74].

The results of the CBMN-Cyt assay indicate that although no significant cytostatic effects were observed, there was notable cytogenetic instability at higher concentrations of ACE and DCM extracts. The significant increase in MNMONO frequencies suggests that these extracts may induce genetic damage, leading to cells failing to complete division. This finding aligns with previous studies that have emphasized the utility of MNMONO in biomonitoring due to its effectiveness in detecting in vivo genetic damage [75,76].

The overall results demonstrate a clear dose–response relationship in the genotoxic effects of ACE, CH, and DCM extracts on human lymphocytes. Specifically, there was a significant increase in the frequency of MNBN, MNMONO, and NPB as the extract concentrations increased. These findings are consistent with the effects observed in the positive control (MMS at 1 mM), indicating that the compounds present in these extracts possess significant genotoxic potential [12,13]. Notably, the DCM extract elevated the frequency of MNBN and MNMONO and induced an increase in NPB, suggesting a broader and more severe genotoxic profile for the compounds extracted with this solvent [77,78].

The observed decrease in the NDI with increasing extract concentrations suggests cytotoxic effects, further highlighting the dual risk these compounds pose to cellular health [79,80]. These findings emphasize the importance of considering both cytotoxic and genotoxic effects when assessing the impact of environmental pollutants, especially in high-exposure areas like industrial and mining zones [11,20]. The variability in effects between different solvents indicates that solvent polarity plays a crucial role in their ability to mobilize and extract specific genotoxic compounds, affecting the type and severity of the genetic damage observed [81].

These findings underscore the need for further research to identify the specific compounds responsible for the observed damage and to elucidate the underlying mechanisms. Moreover, the biological and environmental relevance of these results is significant, as human exposure to these solvents or similar compounds could have serious public health implications, especially for vulnerable populations [82,83]. Future studies should focus on assessing these extracts in other biological models and under chronic exposure conditions to obtain a more comprehensive understanding of their risks.

The detection of CREST+ MN at the highest concentrations of ACE and DCM extracts suggests that these extracts contain substances capable of inducing aneuploidy. This indicates potential disturbances during mitosis, which are possibly due to centromere or kinetochore malfunction or disruption of the mitotic spindle, microtubule assembly, or centrosome [84,85]. The polar and moderately polar characteristics of the substances in these extracts, including oxides and metals such as S, Cr, and Cu, could contribute to these effects. Studies have shown that Cr (VI), in particular, is aneugenic as measured by both chromosome assays and centromere-positive micronuclei assays [86].

Overall, the findings underscore the importance of including both MNBN and MNMONO in biomonitoring studies to capture a broad spectrum of genetic damage indicators. The ability of ACE and DCM extracts to induce aneuploidy and cytogenetic instability points to the need for further investigation into the specific components of these extracts and their potential health implications.

5. Conclusions

Our findings demonstrate that the $PM_{2.5}$ organic fraction collected from the open-pit coal mining area in northern Colombia is primarily composed of modified hydrocarbons and volatile organic compounds, particularly alkenes. The study provides evidence of the cytotoxic and genotoxic effects of these fractions on human lymphocytes, as shown through the significant increases in micronuclei formation, NPB, and oxidative DNA damage, particularly from the ACE and DCM extracts. The Comet and CBMN assays, combined with the use of FPG and ENDO III enzymes, highlighted the oxidative DNA damage caused by these polar fractions. Moreover, the CREST immunostaining confirmed the aneugenic

potential of the ACE and DCM extracts, underlining their capacity to induce chromosomal instability.

These results underscore the importance of monitoring the chemical composition of $PM_{2.5}$ in mining regions to assess potential health risks. The presence of harmful compounds, such as metals and modified PAHs, calls for stricter environmental regulations and public health interventions to minimize exposure. Future studies should focus on identifying the specific compounds responsible for the observed damage and further exploring the mechanisms driving the toxic effects.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/atmos15121420/s1, Figure S1. Results for extractable organic matter (EOM) determination described for sampling sites. Solvents used in Soxhlet extraction are described using colors and acronyms. ACE (acetone); DCM (dichloromethane); CH (cyclohexane).

Author Contributions: Conceptualization, methodology, and supervision, L.E.-P.; C.G.-P., K.P.-S. and P.E.-P.; investigation and methodology, R.E.-S., G.A.-A., E.L.-V., D.S.C. and B.D.-P.; data curation A.H.-Á. and E.L.-V.; software, formal analysis and visualization H.B., D.R.-C. and A.C.-P.; writing—review and editing L.E.-P., J.d.S. and A.P.-T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The Ethics Committee of the University of Sinú approved all the protocols described in this study (Act 002/2012). In addition, authorization and participation in the survey were obtained from the governors of each resguardo, community representatives, and municipal authorities.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available in the article.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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