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Chemical characterisation and cytotoxic effects in A549 cells of urban-air PM10 collected in Torino, Italy.

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(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

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Abstract: Human type II alveolar cell line (A549) was exposed to aqueous and organic solvent PM10 extracts in order to evaluate cell proliferation, proinflammatory cytokines and cytotoxicity (lactate dehydrogenase, LDH). PM10 samples, collected in Torino (north-west Italy), were analyzed for inorganic chemical species (bioavailable iron and secondary particulates) and endotoxins, potentially inflammatory promoters to human airways.

Meanly, in the considered period, PM10 concentration was $55.4 \pm 39.1 \mu\text{g}/\text{m}^3$, iron concentration was 0.078 ± 0.095 and secondary particles constituted $42 \pm 9 \%$ of the PM10 total mass. PM10 inhibits cell proliferation and induces both IL-6 and LDH release in a dose and time dependent manner, with a seasonal trend. The different roles of aqueous and organic solvent PM10 extracts demonstrate the importance of particle composition for the biological effects. Nevertheless few significant correlations were found between the biological effects and PM10 components evaluated in this methods development study.

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Extensive experience in research of cytotoxic effects of air pollution particulate matter in vitro.

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Great knowledge of the evaluation of cytotoxicity and genotoxicity in environmental matrices.

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Great interests in the monitoring and risk assessment of the environmental pollutants.

Opposed Reviewers:

Dear Editor,

We are sending the manuscript "*Cytotoxic effects induced on A549 cells and chemical characterization of urban-air PM10 collected in Torino (Italy)*" that we submit for possible publication on *Environmental Toxicology and Pharmacology*.

PM10 samples were collected in an urban site of Torino, an industrial north-western Italian city, with high levels of particulate matter among European cities. PM10 samples were analyzed for the amount of inorganic chemical species (bioavailable iron and secondary particulates: sulfates and nitrates) and endotoxins, described as potentially inflammatory promoters to human airways. In order to better understand how ambient particulate matter affects lung health, A549 cells were exposed to aqueous and organic solvent PM10 extracts. Effects on cell proliferation, on the release of proinflammatory cytokines and cytotoxicity, measuring lactate dehydrogenase (LDH) release in the extracellular medium, were evaluated.

In general PM10 inhibits cell proliferation and induces both IL-6 and LDH release in a dose and time dependent manner and these biological effects have a seasonal trend. Few significant correlations were found between the biological effects and PM10 components evaluated in this methods development study. Nevertheless the different roles of aqueous and organic solvent PM10 extracts demonstrate the importance of particle composition for the biological effects.

Best regards

Tiziana Schilirò

1 1 **CYTOTOXIC EFFECTS INDUCED ON A549 CELLS AND CHEMICAL**
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3 2 **CHARACTERIZATION OF URBAN-AIR PM10 COLLECTED IN TORINO (ITALY).**

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33 17
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35 18
36
37 19 **Running head:** Biological effects of PM10 induced on A549 cells.

1 28 **Abstract**

2
3 29 Human type II alveolar cell line (A549) was exposed to aqueous and organic solvent
4
5 30 PM10 extracts in order to evaluate cell proliferation, proinflammatory cytokines and
6
7 31 cytotoxicity (lactate dehydrogenase , LDH). PM10 samples, collected in Torino (north-
8
9 32 west Italy), were analyzed for inorganic chemical species (bioavailable iron and
10
11 33 secondary particulates) and endotoxins, potentially inflammatory promoters to human
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13 34 airways.
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16 35 Meanly, in the considered period, PM10 concentration was $55.4 \pm 39.1 \mu\text{g}/\text{m}^3$, iron
17
18 36 concentration was 0.078 ± 0.095 and secondary particles constituted $42 \pm 9 \%$ of the
19
20 37 PM10 total mass. PM10 inhibits cell proliferation and induces both IL-6 and LDH
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22 38 release in a dose and time dependent manner, with a seasonal trend. The different
23
24 39 roles of aqueous and organic solvent PM10 extracts demonstrate the importance of
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26 40 particle composition for the biological effects. Nevertheless few significant correlations
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28 41 were found between the biological effects and PM10 components evaluated in this
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30 42 methods development study.
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44 **Keywords:** Particulate Matter, Cell Proliferation, Cytotoxicity, LDH, Cytokine.

1. Introduction

The health burden due to particulate matter (PM) air pollution is one of the biggest environmental health concerns in the World Health Organization (WHO) European Region and all around the world. PM is a complex and heterogeneous mixture, whose composition (particle size distribution and chemical characteristics) changes in time, space and depends on emissions from various sources, atmospheric chemistry and weather conditions (WHO, 2007).

The breathable fraction of ambient PM is often referred to PM₁₀, defined as particles with a median aerodynamic diameter less than 10 μm , that is a quality air indicator widely measured (WHO, 2006). PM₁₀ may further be divided into two main size fractions, a “coarse” (2.5–10 μm) and a “fine” (0.1–2.5 μm). The *coarse* fraction is dominated by natural sources (geological material: fugitive and resuspended dust; biological material: pollen, endotoxin) and its composition changes depending on the geology of the site considered. The *fine* fraction is dominated by anthropogenic emissions: mixture of carbon particles from combustion processes and secondary particles produced by photochemical reactions in the atmosphere (sulfates and nitrates). The carbonaceous fraction consists of aggregates of organic and inorganic carbon on which are adsorbed transition metals (Pb, Cd, V, Ni, Cu, Zn, Mn, Fe), organic compounds and biological constituents (US EPA, 1996).

Epidemiological and experimental studies have shown that particulate air pollution may induce and aggravate respiratory and cardiovascular diseases. Significant associations between exposure to ambient air particles and increased morbidity and mortality have been demonstrated (Englert 2004; Katsouyanni et al. 2001; Krewski and Rainham 2007; Pope 2007). Most of the available studies do not attribute the observed health effects to a particular characteristic of PM (WHO, 2007). Moreover the exact physiochemical mechanism by which PM produces adverse effects is still poorly

1 81 known; one of the hypotheses considered on PM's mechanisms of action is the
2
3 82 oxidative potential of the particles or specific components.
4
5 83 Several *in vitro* studies with collected particulate matter fractions indicate that particles
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7 84 with a higher oxidative potential have a greater ability to deplete antioxidant defences
8
9 85 and to induce airway inflammation (Ghio et al. 2002; Moller et al. 2008; Schins et al.
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11 86 2004). In this process transition metals such as Fe (Carter et al. 1997; Hutchison et al.
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13 87 2005; Schins et al. 2004), organics such as polycyclic aromatic hydrocarbons (PAHs)
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15 88 (Billet et al. 2008) and endotoxins (Becker et al. 2003; Oberdorster et al. 2000) in PM
16
17 89 seem to be involved (Donaldson et al. 2003; Schwarze et al. 2006; Sorensen et al.
18
19 90 2003). Moreover, although secondary inorganic aerosols have less toxic activity when
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21 91 tested under controlled laboratory conditions, epidemiological studies show significant
22
23 92 associations between sulfates and nitrates and various health outcomes. In ambient
24
25 93 air, this fraction may act as a carrier for other components or as a surrogate for PM
26
27 94 emitted from the combustion of sulfur-containing fuels (Schwarze et al. 2006).
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29
30 95 Recent studies have emphasised the ability of particles to induce cellular responses in
31
32 96 different types of lung cells, as well as inflammation and toxic effects in the whole lung
33
34 97 (Calcabrini et al. 2004; Churg and Brauer 2000; de Kok et al. 2006; Gualtieri et al.
35
36 98 2008; Hetland et al. 2004; Monn and Becker 1999; Shi et al. 2006). *In vitro* studies on
37
38 99 the toxicity of PM10 and its fractions were made by exposing cultures of macrophages
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40 100 and pulmonary epithelial cells to suspension of particles (Becker et al. 2005; Churg and
41
42 101 Brauer 2000). The results of these studies show that exposure to environmental
43
44 102 particles induces cytotoxicity, increase in transcription factors expression (NFkB) and
45
46 103 release in the culture medium of cytokines (such as IL-1, IL- 6, IL-8, MIP-2, GM CSF,
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48 104 TNF) and reactive oxygen species (Becker et al. 2005; Huang et al. 2002; Monn and
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50 105 Becker 1999; Ning et al. 2000). Other *in vitro* studies show that particles can induce
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52 106 damage in plasma membrane and release of cytosolic enzyme lactate dehydrogenase
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1 107 (LDH) (Billet et al. 2007; Garcon et al. 2006; Geng et al. 2006; Molinelli et al. 2002);
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3 108 LDH is considered a marker for acute cytotoxicity (Seagrave and Nikula 2000).
4
5 109 The aim of the present study was to investigate the biological *in vitro* effect of urban-air
6
7 110 PM10 collected in Torino, a north-western Italian city, during two years (2005-2006).
8
9 111 PM10 samples were analyzed for the amount of inorganic species (bioavailable iron
10
11 112 and secondary particulates: sulfates and nitrates) and endotoxins, described as
12
13 113 potentially toxic to human airways. Adherent A549 cells were exposed to PM10
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15 114 aqueous and organic solvent extracts at different concentrations. A549 cells constituted
16
17 115 a useful *in vitro* model for studying human respiratory epithelial cell biology as they
18
19 116 present characteristics similar to human alveolar type II cells (Hatch 1992; Li et al.
20
21 117 2003; Shi et al. 2006; Veronesi et al. 2002) . This study investigated whether different
22
23 118 PM10 extracts could 1) affect the cell proliferation, 2) increase the release of
24
25 119 proinflammatory cytokines e.g. IL-6, and 3) induce cytotoxicity by measuring LDH
26
27 120 release in culture medium. The association of the chemical and *in vitro* toxicological
28
29 121 characterization of urban-air PM10 in one of the most industrialized areas of Italy, with
30
31 122 high levels of particulate matter among European cities (Hazenkamp-Von Arx et al.
32
33 123 2004; Marcazzan et al. 2003) may provide scientific evidences for environmental and
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35 124 sanitary purposes.
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43 126 **2. Materials and Methods**

44 127 *2.1 PM10 sampling*

45 128 PM10 (PM passing through a size-selective inlet with a 50% efficiency cut-off at 10 μ m
46
47 129 aerodynamic diameter) was sampled on glass microfiber filters (Type A/E, 8" x 10",
48
49 130 Gelman Sciences, Michigan, USA), with a Sierra Andersen High Volume Sampler
50
51 131 1200/VFC (Andersen Samplers, Atlanta, Georgia, USA) using a flow of 1160 L/min.
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53 132 Sample duration was controlled by a timer accurate to \pm 15 min over a 24 hr sample
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55 133 period. The exact flow was calculated daily, corrected for variation in atmospheric
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1 134 pressure and actual differential pressure across the filter. The filters were pre- and
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3 135 post- conditioned by moving them to a dry and dark environment for 48h, and they
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5 136 were weighed in a room with controlled temperature and humidity. Procedures were
6
7 137 conducted according to the European Committee for Standardization (CEN, 1998). The
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9 138 PM10 concentration, C ($\mu\text{g}/\text{m}^3$), in the air volume sampled, V (L), was calculated as
10
11
12 139 follows:

$$14 140 C = [(W_2 - W_1) - (B_2 - B_1)] * 10^3 / V$$

16 141 where W_1 is the mean of three tare weights of the same filter before sampling (mg), W_2
17
18 142 is mean of three post-sampling weights of the same sample-containing filter (mg), B_1 is
19
20 143 mean tare weight of blank filters (mg). B_2 is mean post-sampling weight of blank filters
21
22 144 (mg) and V is volume as sampled at the nominal flow rate.

25 145 Samplings were carried out from January 2005 to December 2006 (2 filters every
26
27 146 month, one sampled at the half and one at the end of every month, without considering
28
29 147 week-ends) in a meteorological-chemical background station located in the urban
30
31 148 centre of Torino, an industrial north-western Italian city (Gilli et al. 2007b).

34 149 *2.2 Particles extractions*

36 150 Each PM10 filter (typically 20.32 cm by 25.40 cm) was treated individually: two different
37
38 151 6.35 cm by 20.32 cm wide strips were cut from the same filters and each portion has
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40 152 been used with different extraction medium: acetone and the Dulbecco's Modified
41
42 153 Eagle's Medium (DMEM) culture medium without fetal calf serum (FCS). Acetone was
43
44 154 chosen as solvent to extract organic-extractable compounds (Claxton et al. 2004).
45
46 155 DMEM without FCS was chosen to extract water-soluble components, hypothetically
47
48 156 comparable to the extraction at the lung cells (Calcabrini et al. 2004; Hetland et al.
49
50 157 2004). Each portion of the filter has been cut into little strips and placed in 50 mL
51
52 158 polypropylene sterile tube with 15 mL of the extraction medium. The tubes were placed
53
54 159 in an ultrasonic water bath for 10 min followed by 1 min of vortexing. This procedure
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56 160 was repeated 3 times. The samples were centrifugated at 5000 rpm for 10 min to
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1 161 remove the filter material and the supernatant was collected. Acetone extracts were
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3 162 evaporated with a rotary evaporator and re-suspended in culture medium. Aqueous
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5 163 extracts were directly assayed. It was assumed that particles were totally removed from
6
7 164 the filters through extraction procedures (Hutchison et al. 2005). Unless specified
8
9 165 otherwise, all chemicals were purchased from Sigma, USA.

166 *2.3 Iron and bioavailable iron determinations*

167 Iron determination was performed according to the procedure of Gilli et al. (2007b).
168 Briefly, the metals were extracted from filter strips (3.18 cm by 20.32 cm) by a nitric
169 acid solution. After cooling, the sample was mixed and centrifuged and the trace
170 element concentrations in each sample were determined by atomic absorption
171 spectrometry, Varian GTA-96.

172 The bioavailable iron from urban particulates was determined as previously described
173 (Lund and Aust 1990), with some modifications as reported by (Smith and Aust 1997)
174 and Gilli et al. (2007b). Briefly, filter strips (3.18 cm by 20.32 cm) were suspended in
175 NaCl 50nM, mixed and pH adjusted to 7.5. Citrate was then added to samples to obtain
176 a final concentration of 1 mM and all samples were placed on a wrist-action shaker in
177 the dark for 24 h. One mL samples were withdrawn and centrifuged to remove the
178 particulates. The amount of iron mobilized as the citrate: Fe complex in the supernatant
179 was determined using a spectrophotometric total non-heme iron assay (Brumby and
180 Massey, 1967). The concentration of iron mobilized by citrate was expressed as μg of
181 Fe/m^3 and as ng of $\text{Fe}/\mu\text{g}$ of particulate.

182 *2.4 Sulfates and nitrates determination*

183 Sulfates and nitrates determination was performed according to the procedure of Gilli
184 et al. (2007a, 2007b). Briefly each filter strip (3.18 cm by 20.32 cm) was extracted in 15
185 mL in distilled deionized waters, via 30 min sonication, 30 min agitation and an
186 overnight refrigeration at 4°C. Prior to the analysis, the samples were centrifuged to
187 remove particles. Ion chromatography (IC) was used to determine the soluble ion

1 188 content sulfates (SO_4^-) and nitrates (NO_3^-), applying a Dionex DX-100 ion
2
3 189 chromatograph with NaHCO_3 0.3 mM and Na_2CO_3 2.7 mM for eluent and IonPac
4
5 190 analytical column S12A for anions. Both the utilized standards, sodium sulfate and
6
7 191 sodium nitrate (71959 and 71759 FLUKA respectively) ranged from 0.1 $\mu\text{g}/\text{mL}$ to 100
8
9 192 $\mu\text{g}/\text{mL}$. The ions were identified by their elution/retention times (about 8.75 min and
10
11 193 12.5 min for nitrates and sulfates respectively) and quantified by the conductivity peak
12
13 194 area or peak height (300A method, US EPA, 1996).

16 195 *2.5 Endotoxins determination*

18 196 Endotoxin was assayed with an end-point chromogenic Limulus ameocyte lysate
19
20 197 (LAL) method (QLC-1000 n° 50-648U, Cambrex, Walkersville, MD, USA) at 37°C with
21
22 198 an automated micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc) following
23
24 199 manufacturer's instructions. *Escherichia coli* 0111:B4 endotoxin was used as standard
25
26 200 endotoxin. Sample concentration were reported as endotoxin units (EU) per millilitre of
27
28 201 eluant, EU per milligram of PM10, and EU per cubic meter of air collected. The limit of
29
30 202 detection (LOD) was 0.01 EU/mL. To control for the potential interference of samples
31
32 203 substances with the LAL assay, all samples were spiked with a known amount of
33
34 204 endotoxin (0.4 EU/mL). The spiked solution was assayed along with the unspiked
35
36 205 samples and their respective endotoxin concentrations are determined. The difference
37
38 206 between these two calculated endotoxin values should equal the known concentration
39
40 207 of the spike $\pm 25\%$ to accept the results of the determinations.

45 208 *2.6 Cell culture*

47 209 The human lung epithelial cell line A549 (non-small cell lung cancer) from Interlab Cell
48
49 210 Line Collection (Genova, IT) was used as a model for human epithelial lung cells. Cells
50
51 211 were grown as a monolayer, maintained and treated in DMEM supplemented with 10%
52
53 212 FCS, 2% L-glutamine 200mM, 2% HEPES 1M, 1% sodium pyruvate 100 mM and 1%
54
55 213 penicillin/streptomycin 10mg/mL, at 37°C in an humidified atmosphere containing 5%
56
57 214 CO_2 .

2.7 Cell proliferation

Cell proliferation was evaluated using crystal violet method (Kueng et al. 1989). Cells were seeded in 24-well plates at a density of 4×10^4 cell/well and exposed to particle concentration of 50 and 100 $\mu\text{g/mL}$ (30 and 60 $\mu\text{g/cm}^2$), (Alfaro-Moreno et al. 2002; Calcabrini et al. 2004; Veronesi et al. 2002). Cell proliferation was determined at 24h, 48h and 72h of exposure measuring the residual cell number by crystal violet by determining the absorbance at 595 nm with a micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc). At the same time blank filters were treated in the same way. All experiments were performed in triplicate. Percentage cell proliferation was calculated comparing the absorbance of exposed cultures with the absorbance of non-exposed cultures.

2.8 Proinflammatory cytokine in cell supernatant

IL-6 is a multifactorial cytokine protein that plays a major role in the mediation of airways inflammation associated with infection or toxic insult. A549 cells were exposed to particle concentration of 50 and 100 $\mu\text{g/mL}$ (30 and 60 $\mu\text{g/cm}^2$) and after 24, 48 and 72h of treatment, supernatant aliquots were removed for the cytokine assay. IL-6 was measured in the culture supernatant by enzyme-linked immunoabsorbent assay (ELISA) kits (PeliPair reagent set, Sanquin, Netherlands) with an automated micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc) according to the manufacturer's recommendations. Results were calculated by expressing samples IL-6 increment as a percentage of IL-6 expression from non exposed cells.

2.9 LDH assay

To evaluate PM10 cytotoxicity, released lactate dehydrogenase (LDH) activities, from damaged cells, were measured in cell-free culture supernatants. Enzymatic activities, measured spectrophotometrically as absorbance variation at 340 nm (37°C), were expressed as nmol NADH oxidized/min in the conversion of pyruvate to lactate (Golladay et al. 1997; Kinnula et al. 1994; Riganti et al. 2002). A549 cells were seeded

1 242 in 6 well plates at a density of 1×10^6 cell/well and exposed to PM10 extracts containing
2
3 243 50, 100 and 200 $\mu\text{g/mL}$ of particles (30, 60 and $110 \mu\text{g/cm}^2$), (Billet et al. 2007; Geng et
4
5 244 al. 2006; Hetland et al. 2000). At the same time blank filters were treated in the same
6
7 245 manner. At 24h, 48h and 72h LDH activity was measured in supernatant and cell
8
9 246 lysate. LDH activity was calculated as ratio between the extracellular LDH (measured
10
11 247 in the supernatant) and total LDH (expressed as sum of LDH measured in supernatant
12
13 248 and cell lysate). To obtain cell lysate, cells were washed with PBS, detached with
14
15 249 trypsin-EDTA, resuspended with 1 mL of TRAP (82.3 mM triethanolamine
16
17 250 hydrochloride, pH 7.6) and sonicated for 10". Then LDH was measured by adding 250
18
19 251 μl of mix containing NADH 0.25 mM and pyruvate 0.5 mM and consumption of NADH
20
21 252 was measured as absorbance in a microplate reader (Benchmark Plus Microplate
22
23 253 Reader, Biorad) at 340 nm. All experiments were performed in triplicate. Results were
24
25 254 calculated by expressing samples LDH activity as a percentage of LDH activity from
26
27 255 non exposed cells.

256 2.10 Statistical analysis

257 Statistical analyses were performed using the SPSS Package, version 14.0 for
258 Windows. T-test analysis was used to compare means and Spearman correlation
259 coefficient was used to assess relationship between variables. The mean difference
260 and correlation were considered significant at $p < 0.05$.

261 3. Results

262 3.1 PM10 concentration

263 A total of 46 PM10 filters were analyzed during 2005 and 2006 without considering
264 week-ends. The mean PM10 concentration during the whole sampling period was 55.4
265 $\pm 39.1 \mu\text{g/m}^3$. The highest value was observed in winter ($198.5 \mu\text{g/m}^3$), while the lowest
266 was observed in summer ($12.4 \mu\text{g/m}^3$); the T-test analysis show significant ($p < 0.01$)
267

1 268 difference for the seasonal (autumn/winter vs spring/summer) PM10 concentrations
2
3 269 (Table 1).

4 5 270 *3.2 Iron and bioavailable iron concentrations*

6
7 271 The total iron mean concentration was $1.035 \pm 0.811 \mu\text{g}/\text{m}^3$ and, in relation to PM10,
8
9 272 $19.43 \pm 11.64 \text{ ng}/\mu\text{g}$ particles. The highest value was observed in winter ($3.751 \mu\text{g}/\text{m}^3$),
10
11 273 while the lowest was observed in summer ($0.099 \mu\text{g}/\text{m}^3$). A statistically significant
12
13 274 difference was found for the seasonal concentrations (autumn/winter vs
14
15 275 spring/summer) expressed as $\mu\text{g}/\text{m}^3$ (t-test, $p < 0.01$) but not as $\text{ng}/\mu\text{g}$ particles (Table
16
17 276 2). A significant positive correlation was found with PM10 concentrations (Spearman
18
19 277 correlation: $r = 0.800$, $p < 0.01$).

20
21 278 Table 2 shows also the mean concentrations of bioavailable iron, $6.9 \pm 4.5\%$ of total
22
23 279 iron was bioavailable. No statistically significant differences were found for the mean
24
25 280 seasonal concentrations expressed both as $\mu\text{g}/\text{m}^3$ (t-test, $p < 0.01$) and as $\text{ng}/\mu\text{g}$
26
27 281 particles. A positive significant correlation was found with PM10 concentrations
28
29 282 (Spearman correlation: $r = 0.670$, $p < 0.01$).

30 31 283 *3.3 Sulfates and nitrates concentrations*

32
33 284 The sulfates mean concentration was $10.5 \pm 5.7 \mu\text{g}/\text{m}^3$ and, in relation to PM10, 216.7
34
35 285 $\pm 72.7 \text{ ng}/\mu\text{g}$ particles, such that sulfates represented $21.2 \pm 6.9\%$ of total PM10 mass.
36
37 286 The nitrates mean concentration was $11.7 \pm 9.2 \mu\text{g}/\text{m}^3$ and, in relation to PM10, 216.3
38
39 287 $\pm 80.5 \text{ ng}/\mu\text{g}$ particles, such that nitrates represented $20.6 \pm 6.1\%$ of total PM10 mass.
40
41 288 Considering the sum of sulfates and nitrates as principal components of secondary
42
43 289 particulate, it was found that they meanly constituted $42 \pm 9\%$ of the PM10 total mass
44
45 290 (Table 1). A statistically significant difference was found for the seasonal
46
47 291 concentrations for both sulfates and nitrates expressed as $\mu\text{g}/\text{m}^3$ (t-test, $p < 0.05$ and p
48
49 292 < 0.01 , respectively). Considering the concentrations of the two species in relation to
50
51 293 PM10 (percentage), it was found that in the hot season the sulphate amount ($23.6 \pm$
52
53 294 5.6%) was more elevated than in the cold season ($19.2 \pm 7.3\%$) and this trend was

1 295 significant (t-test, $p < 0.01$), while for nitrates the amount was similar in the two
2
3 296 seasons. A significant positive correlation was found with PM10 concentrations both for
4
5 297 sulfates (Spearman correlation: $r=0.740$, $p<0.01$) and for nitrates (Spearman
6
7 298 correlation: $r=0.900$, $p<0.01$) and also a significant positive correlation between sulfates
8
9 299 and bioavailable iron (Spearman correlation: $r=0.490$, $p<0.01$).

10 300 *3.4 Endotoxins concentrations*

11 301 Endotoxin mean concentration during the whole sampling period was 0.19 ± 0.07
12
13 302 EU/m³ and in relation to PM10 3.70 ± 0.95 EU/mg. A statistically significant difference
14
15 303 was found for the seasonal concentrations (autumn/winter vs spring/summer)
16
17 304 expressed as EU/m³ (0.24 ± 0.07 vs 0.14 ± 0.01 respectively) (t-test, $p<0.05$) but not as
18
19 305 EU/mg particles. Endotoxins, EU/mg, were inversely correlated with PM10
20
21 306 concentrations (Spearman correlation: $r = - 0.710$, $p < 0.05$).

22 307 *3.5 A549 cell proliferation*

23 308 Mean absorbance values at 595 nm of the negative control at 24, 48 and 72 h were
24
25 309 respectively 0.120 ± 0.010 , 0.225 ± 0.010 and 0.410 ± 0.015 . Extracts from blank filters
26
27 310 had no significant effect on cell proliferation. Figure 1 shows effects of organic solvent
28
29 311 (1a) and aqueous (1b) PM10 extracts on cell proliferation. PM10 inhibits cell
30
31 312 proliferation in a dose and time dependent manner. Aqueous extracts seem to have
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33 313 stronger effects than organic solvent extracts on cell proliferation, this trend was
34
35 314 confirmed by T-tests for inhibition at 48 and 72 h ($p < 0.05$) but not at 24 h, for both the
36
37 315 tested concentrations. The maximum inhibition of cell proliferation, 25.3 ± 5.8 %, was
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39 316 achieved at 72h by the 100 $\mu\text{g/mL}$ aqueous extract.

40 317 Proliferation inhibition of A549 cells due to organic solvent extract, in some cases (50
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42 318 $\mu\text{g/mL}$ at 72h, 100 $\mu\text{g/mL}$ at 48 and 72h) was correlated with PM10 concentrations
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44 319 (Spearman correlations: $r=0.860$, $p<0.01$; $r=0.810$, $p<0.01$ and $r=0.620$, $p<0.05$
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1 320 respectively), no correlations were found with any other parameters or for the aqueous
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3 321 extracts.

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5 322 Figure 3(a) shows different seasonal (autumn/winter vs spring/summer) proliferation
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7 323 inhibition of A549 cells at 72h after their incubation in the continuous presence of two
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9 324 concentrations of particle matter (50 and 100 µg/mL). Despite PM10 organic solvent
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11 325 extracts and PM10 aqueous extracts always had higher effect in the cold months these
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13 326 differences were not significant (T-tests $p > 0.05$).

14 327 *3.6 IL-6 release in A549 cells*

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18 328 In order to investigate the proinflammatory potential of the particulate, the production of
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20 329 IL-6 in the presence of two different extracts of PM10 was analyzed. Mean IL-6 release
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22 330 in the negative control at 24, 48 and 72h range from 0.2 to 1.0 pg/mL. PM10 extracts
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24 331 induce IL-6 release in a dose and time dependent manner. As shown in figure 2a, the
25
26 332 organic solvent extract induce significant release of IL-6 in relation to the control at 48h
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28 333 and 72h of exposure (T-test $p < 0.05$) for both the concentrations, the lowest
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30 334 concentration at 24h of exposure has no significant increase of IL-6 release in relation
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32 335 to the control ($6.2 \pm 3.5\%$). The two tested concentrations exhibited similar expression
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34 336 of cytokine without significant difference (t-test $p > 0.05$).

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38 337 In figure 2b, the aqueous extract induce significant release of IL-6 in relation to the
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40 338 control at 24h, 48h and 72h of exposure (T-test $p < 0.05$) for cells exposed to 100
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42 339 µg/mL while for cells exposed to 50 µg/ml only at 72h.

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45 340 Despite the aqueous extract exhibit higher mean levels of IL-6 expression the
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47 341 difference between the production of IL-6 in the presence of organic solvent extract or
48
49 342 aqueous extract was not statistically significant (T-test $p > 0.05$) for any concentrations
50
51 343 or times. The maximum increment of IL-6 release, $108.3 \pm 39.1\%$, was obtained at 72h
52
53 344 by the 100 µg/mL aqueous extract.

1 345 IL-6 release by A549 due to organic solvent extracts (50 and 100 µg/mL at 72h) was
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3 346 correlated with PM10 concentrations (Spearman correlations: $r=0.680$, $p<0.05$ and
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5 347 $r=0.730$, $p<0.05$ respectively). No correlations were found with any other parameters.
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7 348 IL-6 release by A549 due to aqueous extracts was always correlated with PM10,
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9 349 sulfate, nitrate and Fe concentrations for both the extract concentrations and at each
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11 350 time of exposure in a range of Spearman correlation between $r = 0.720$ to $r = 0.920$,
12
13 351 $p<0.05$.

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15
16 352 Figure 3(b) shows different seasonal (autumn/winter vs spring/summer) IL-6 release at
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18 353 72h after their incubation in the continuous presence of two concentrations of particle
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20 354 matter (50 and 100 µg/mL). PM10 organic solvent extracts and PM10 aqueous extracts
21
22 355 always had higher effect in the cold months and these differences were statistically
23
24 356 significant (t-test, $p < 0.01$) except for one case (50µg/mL organic solvent extract).

27 357 *3.7 LDH release in A549 cells*

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29 358 An increase of extracellular LDH enzyme activity reflects an increase in the amount of
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31 359 membrane-damage cells (Lobner 2000). The release of the cytoplasmatic enzyme LDH
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33 360 into the culture supernatant was used to measure cell cytotoxicity. Extracts from blank
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35 361 filters had no significant effect on LDH release. Incubation of A549 cells with particles
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37 362 at a concentration of 50 µg/mL had no effect on LDH release as compared to control
38
39 363 cells (data not shown). Results are shown in table 3: for organic solvent extract and for
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41 364 both the concentrations the highest and significant release of LDH was obtained at 72h
42
43 365 of exposure (T-tests, $p < 0.05$), while at 24h and 48h no significant effects were
44
45 366 observed in relation to the control (T-tests, $p > 0.05$). For aqueous extracts exposure to
46
47 367 100 µg/mL of particles increase LDH release by approximately 30-35% for all
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49 368 incubation time. Statistically significant increase of extracellular LDH activities were
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51 369 observed at 24h, 48h and 72h after cell exposure at 200 µg/mL of particles
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53 370 concentration (T-tests, $p < 0.05$). The maximum increment of LDH release, 87.6 ± 41.9
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1 371 % and 83.7 ± 44.1 %, was respectively obtained at 48h by the 200 $\mu\text{g}/\text{mL}$ aqueous
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3 372 extract and at 72h by the 200 $\mu\text{g}/\text{mL}$ organic solvent extract .
4
5 373 LDH increase by A549 due to both organic solvent extracts and aqueous extracts was
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7 374 never correlated with PM10 concentrations or with any other parameters, with the
8
9 375 exception of the aqueous 200 $\mu\text{g}/\text{mL}$ extract at 72h vs PM10 concentrations.
10
11 376 Figure 3(c) shows different seasonal (autumn/winter vs spring/summer) release of LDH
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13 377 activity from A549 cells at 72h after their incubation in the continuous presence of two
14
15 378 concentrations of particle matter (100 and 200 $\mu\text{g}/\text{mL}$). PM10 organic solvent extracts
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17 379 had similar effect in the two seasons while PM10 aqueous extracts always had higher
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19 380 effect in the cold months, this difference was significant only for the lowest extract
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21 381 concentration.
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27 383 **4. Discussion and Conclusions**

28 384 The PM10 monitoring in Torino has showed concentrations of PM10 often over the
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30 385 daily ($50 \mu\text{g}/\text{m}^3$) and yearly ($40 \mu\text{g}/\text{m}^3$) quality targets (Air Quality Directive,
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32 386 2008/50/CE). High pollution episodes are frequent during winter time in Northern Italy.
33
34 387 This is due to the regional unique climate and topography which hamper particles
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36 388 dispersion. Human alveolar epithelial cells were exposed to concentration of particles
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38 389 comparable to $100 \mu\text{g}/\text{m}^3$ such as those found in winter season in Torino, indeed the
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40 390 averages were $93.5 \pm 54.1 \mu\text{g}/\text{m}^3$ for 2005 and $78.4 \pm 53.1 \mu\text{g}/\text{m}^3$ for 2006 (Oberdörster
41
42 391 and Yu 1999).
43
44 392 In urban sites like that utilized in this study, significant seasonal differences in total Fe
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46 393 concentrations occur probably due to minor presence of anthropogenic sources (for
47
48 394 example domestic and industrial heating) in the warm season (Gilli et al. 2007b;
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50 395 Ziemacki et al. 2003). A small amount of iron is presumably bioavailable (about 6% of
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52 396 total Fe) and this form is capable of generating ROS and induces inflammation in
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1 397 cellular systems (Aust et al. 2002). In this study this bioavailable Fe has not a
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3 398 significant seasonal trend and it is less correlated with PM10 concentration than total
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5 399 Fe.
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7 400 In relation to secondary PM10 components, the seasonal trend of the two species is
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9 401 normally due to the photochemical reactions that, in warm season occur more
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11 402 frequently. It was found that the secondary airborne particulate matter (as sum of
12
13 403 sulfates and nitrates) mass concentration was about 42% of the total PM10, and this
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15 404 value was confirmed by other studies conducted in the Po valley of Northern Italy (Gilli
16
17 405 et al. 2007b; Marcazzan et al. 2003).
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19 406 Among relevant air pollution components in causing toxicity there are also endotoxins;
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21 407 in this study the mean endotoxins concentration was low and quite comparable with
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23 408 those present in literature (Heinrich et al. 2003; Morgenstern et al. 2005; Mueller-
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25 409 Anneling et al. 2004; Solomon et al. 2006).
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27 410 Many studies have investigated the toxicity and mechanism of PM in the airway
28
29 411 epithelial cells (Scapellato and Lotti 2007); PM induces different biologic effects
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31 412 depending on sampling site (Rosas-Pérez et al., 2007), size fraction (Alfaro-Moreno et
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33 413 al. 2002; Hetland et al. 2004; Osornio-Vargas et al. 2003), sampling time (Frampton et
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35 414 al. 1999) and contaminants adsorbed on the particles (Baulig et al. 2003; Billet et al.
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37 415 2007; Calcabrini et al. 2004; Muller et al. 2006). Moreover all in vitro studies with PM10
38
39 416 usually used high concentration of particles so that a potential confounding effect of
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41 417 high doses need to be taken into account when extrapolating to *in vivo* conditions
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43 418 (Oberdorster and Yu 1999).
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45 419 In this study PM10 inhibits cell proliferation and induces both IL-6 and LDH release in a
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47 420 dose and time dependent manner according to other studies on epithelial cells (Alfaro-
48
49 421 Moreno et al. 2002; Osornio-Vargas et al. 2003). These biological effects have a
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51 422 seasonal trend as well as other biological effects such as mutagenicity evaluated in a
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53 423 study conducted in the Torino city (Gilli et al. 2007a).
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1 424 In general the maximum biological effect on A549 cells was obtained in term of IL-6
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3 425 release (108.3 ± 39.1 %), then LDH production (87.6 ± 41.9 %) and in the end inhibition
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5 426 of cell proliferation (25.3 ± 5.8 %). Indeed other studies have shown that PM10 induce
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8 427 - first the production of reactive oxygen species and inflammatory mediators such as
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10 428 interleukin IL-6 - than acute cytotoxicity as release of LDH and in the end - an alteration
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12 429 of the cellular function highlighted by inhibition of the cellular proliferation (Jalava et al.
13
14 430 2006). It is also important to consider that these outcomes might occur independently
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16 431 or in association with each others (Seagrave and Nikula 2000).
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18 432 In our investigation aqueous extracts seem to induce a greater IL-6 release and a
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20 433 greater proliferation inhibition than organic solvent (acetone) ones, while, on the
21
22 434 contrary, organic solvent extract induce a greater release of LDH. Probably ,although
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24 435 both the two PM10 extracts produce effects on cells, water-soluble components, such
25
26 436 as transition metals, could rapidly cross the plasma membrane and induce the
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28 437 inflammatory response, while polar compounds, from organic solvent extracts, cross
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30 438 the plasma membrane more slowly and are able to directly interact with DNA and
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32 439 induce mutations (Gilli et al. 2007a; Gilli et al. 2007b).
33
34 440 In general the biological effects are frequently attributed to the intracellular generation
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36 441 of ROS, usually correlated with aqueous PM extracts, that may damage protein
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38 442 structure, plasmatic membrane associated lipids and other cellular compartments
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40 443 (Knaapen et al. 2004; Sevanian and Ursini 2000; Sorensen et al. 2003). Soukup and
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42 444 Becker (Soukup and Becker 2001) found that expression of cytokines by human
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44 445 alveolar macrophages incubated with ambient insoluble PM10 was more than 50 times
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46 446 higher than that caused by the water-soluble fraction. Another study, conducted by
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48 447 Javala et al. (2008), is focused on the stronger cytotoxic effects of insoluble (organic)
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50 448 than water-soluble fraction of PM. In another investigation, mRNA levels of IL-1 α and
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52 449 IL-8 were significantly more expressed by human bronchial epithelial cells when
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1 450 incubated with the water-soluble fraction of ambient PM10 than with the corresponding
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3 451 suspensions (10–500 µg/mL, up to 24 h) (Fujii et al. 2001). To our knowledge, few
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5 452 studies in literature compare the different effects of urban PM10 extracts.
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7 453 Few significant correlations were found between the biological effects and PM10
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9 454 components (bioavailable iron, secondary particulates and endotoxins) evaluated in
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11 455 this methods development study and it is important to note that the correlations have
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13 456 become significant only after some days of exposure (72h). These results confirmed
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15 457 other recent studies in which, for *in vitro* lung cells exposure, there were inconsistent
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17 458 associations between both cell proliferation and IL-6 release and secondary
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19 459 particulates or metals (Happo et al. 2008). One possible explanation for the presence
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21 460 of few correlations could be that the biological end-points and the PM10 components
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23 461 were quantified in chemically separate fractions. Furthermore the aqueous extracts did
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25 462 not reflected the activity of any particular PM10 chemicals components but only
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27 463 highlighted the activity of physiologic-soluble components.
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29 464 Nevertheless the significant difference in biological effects induced by the two different
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31 465 extracts (aqueous and organic solvent) and the observed seasonal trend, support the
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33 466 need of further studies, in order to determine particle components responsible for
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35 467 biological effects associated both to PM10 and fine particulate matter.
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37 468 The identification of PM components carrying the relevant burden in causing
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39 469 inflammation remains unsettled, and it was said to represent one of the biggest gaps in
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41 470 our knowledge (Pope and Dockery 2006).
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43 471 In the future, better understanding of the relative toxicity and health effects of particles
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45 472 from various sources could facilitate targeted abatement policies and more effective
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47 473 control measures to reduce the burden of disease due to air pollution.
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16 720 **Figure Captions**

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21 722 **Figure 1.** Proliferation inhibition of A549 cells after their incubation in the continuous
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23 723 presence of increasing concentrations of particulate matter (50 and 100 µg/mL) from
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25 724 PM10 organic solvent extracts (a) and PM10 aqueous extracts (b). Inhibition of cell
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27 725 proliferation was calculated comparing the absorbance of exposed cultures with the
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29 726 absorbance of non-exposed cultures. Asterisks indicate statistically significant
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31 727 differences from the control , $p < 0.05$ (T-test).

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36 729 **Figure 2.** IL-6 release by A549 cells after their incubation in the continuous presence of
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38 730 increasing concentrations of particulate matter (50 and 100 µg/mL) from PM10 organic
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40 731 solvent extracts (a) and PM10 aqueous extracts (b). Results were calculated by
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42 732 expressing samples IL-6 relies as a percentage of IL-6 release from non exposed cells.
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44 733 Asterisks indicate statistically significant differences from the control , $p < 0.05$ (T-test).

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49 735 **Figure 3.** Different seasonal (autumn/winter vs spring/summer) proliferation inhibitions
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51 736 3(a), IL-6 releases 3(b) and LDH releases 3(c) from A549 cells at 72h after their
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53 737 incubation in the continuous presence of PM10 organic solvent extracts and PM10
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55 738 aqueous extracts (50, 100 µg/mL or 200 µg/mL). Asterisks indicates statistically
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57 739 significant differences for the two seasons, $p < 0.05$ (T-test).



Environmental Toxicology and Pharmacology

Conflict of Interest Policy

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Giorgio Gilli

Declarations

Environmental Toxicology and Pharmacology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

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Print name

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Table 1. Means and standard deviations of PM10, sulfates and nitrates concentrations ($\mu\text{g}/\text{m}^3$) of the whole sampling period (2005/2006) and divided by seasons.

	PM10 ($\mu\text{g}/\text{m}^3$)	SO₄⁼ ($\mu\text{g}/\text{m}^3$)	NO₃⁻ ($\mu\text{g}/\text{m}^3$)	SO₄⁼ (% of PM10 mass)	NO₃⁻ (% of PM10 mass)
Whole period	55.4 ± 39.1	10.5 ± 5.7	11.7 ± 9.2	21.2 ± 6.9	20.6 ± 6.1
Winter/Autumn	71.3 ± 43.1**	12.4 ± 6.4*	14.9 ± 10.1**	19.2 ± 7.3*	20.8 ± 6.0
Summer/Spring	37.4 ± 24.1**	8.3 ± 5.7*	8.0 ± 6.3**	23.6 ± 5.6*	20.3 ± 6.3

** statistically significant differences, (autumn/winter vs spring/summer) $p < 0.01$ (T-test).

* statistically significant differences, (autumn/winter vs spring/summer) $p < 0.05$ (T-test).

Table 2. Means and standard deviations of Fe concentrations ($\mu\text{g}/\text{m}^3$) of the whole sampling period (2005/2006) and divided by seasons.

	Fe total ($\mu\text{g}/\text{m}^3$)	Fe bio ($\mu\text{g}/\text{m}^3$)	% Fe bio (of total)
Whole sampling period	1.035 \pm 0.811	0.078 \pm 0.095	6.9 \pm 4.5
Winter/Autumn	1.355 \pm 0.919**	0.097 \pm 0.089	7.5 \pm 4.8
Summer/Spring	0.672 \pm 0.463**	0.056 \pm 0.099	6.2 \pm 4.1

** statistically significant differences, (autumn/winter vs spring/summer) $p < 0.01$ (T-test).

Table 3. Released lactate dehydrogenase (LDH) activity from A549 cell 24h, 48h and 72h after their incubation in the continuous presence of increasing concentrations of particle matter (100 and 200 µg/mL) from PM10 organic solvent extracts and from PM10 aqueous extracts. Results were calculated by expressing samples LDH activity as a percentage of LDH activity from non exposed cells.

LDH increase %		24 h	48 h	72 h
PM10 organic solvent extracts	100 µg/mL	-2.7 ± 7.9	3.9 ± 7.1	64.0 ± 22.5*
	200 µg/mL	-7.3 ± 3.1	2.9 ± 5.5	83.7 ± 44.1*
PM10 aqueous extracts	100 µg/mL	30.2 ± 24.3	33.5 ± 23.0*	35.6 ± 18.3*
	200 µg/mL	78.1 ± 34.1*	87.6 ± 41.9*	53.6 ± 24.6*

* statistically significant differences in relation to the control, $p < 0.05$ (T-test).

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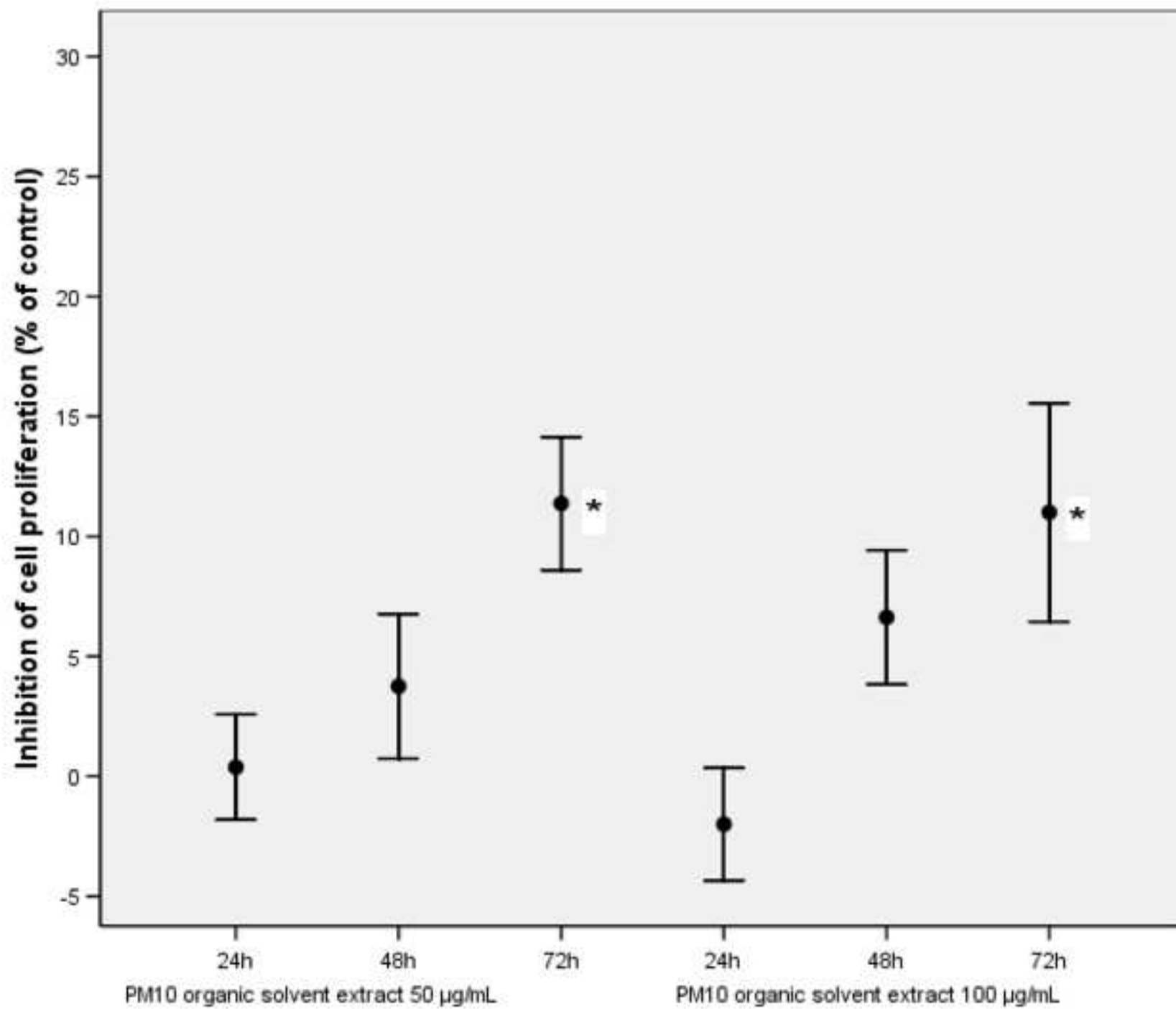


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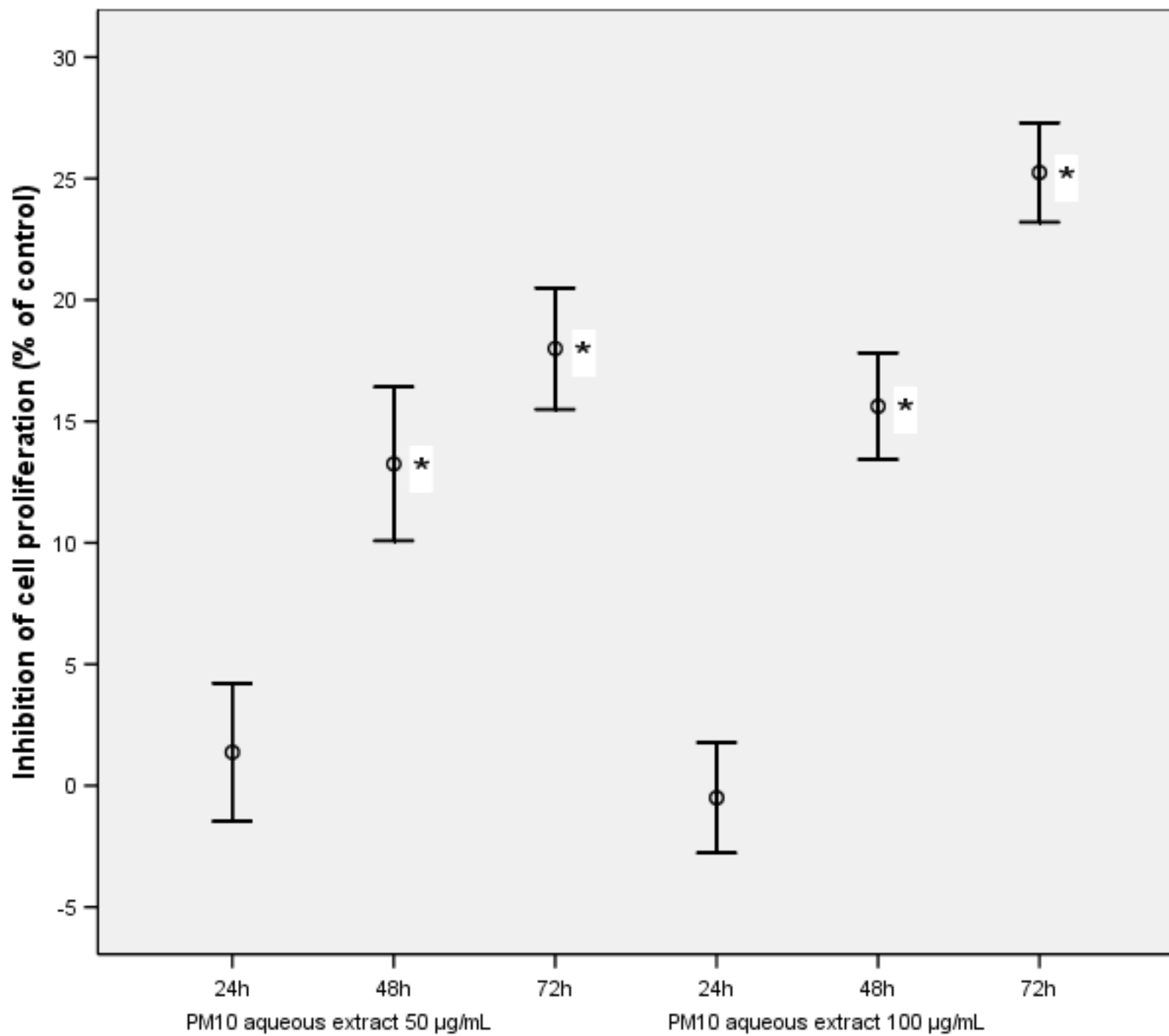


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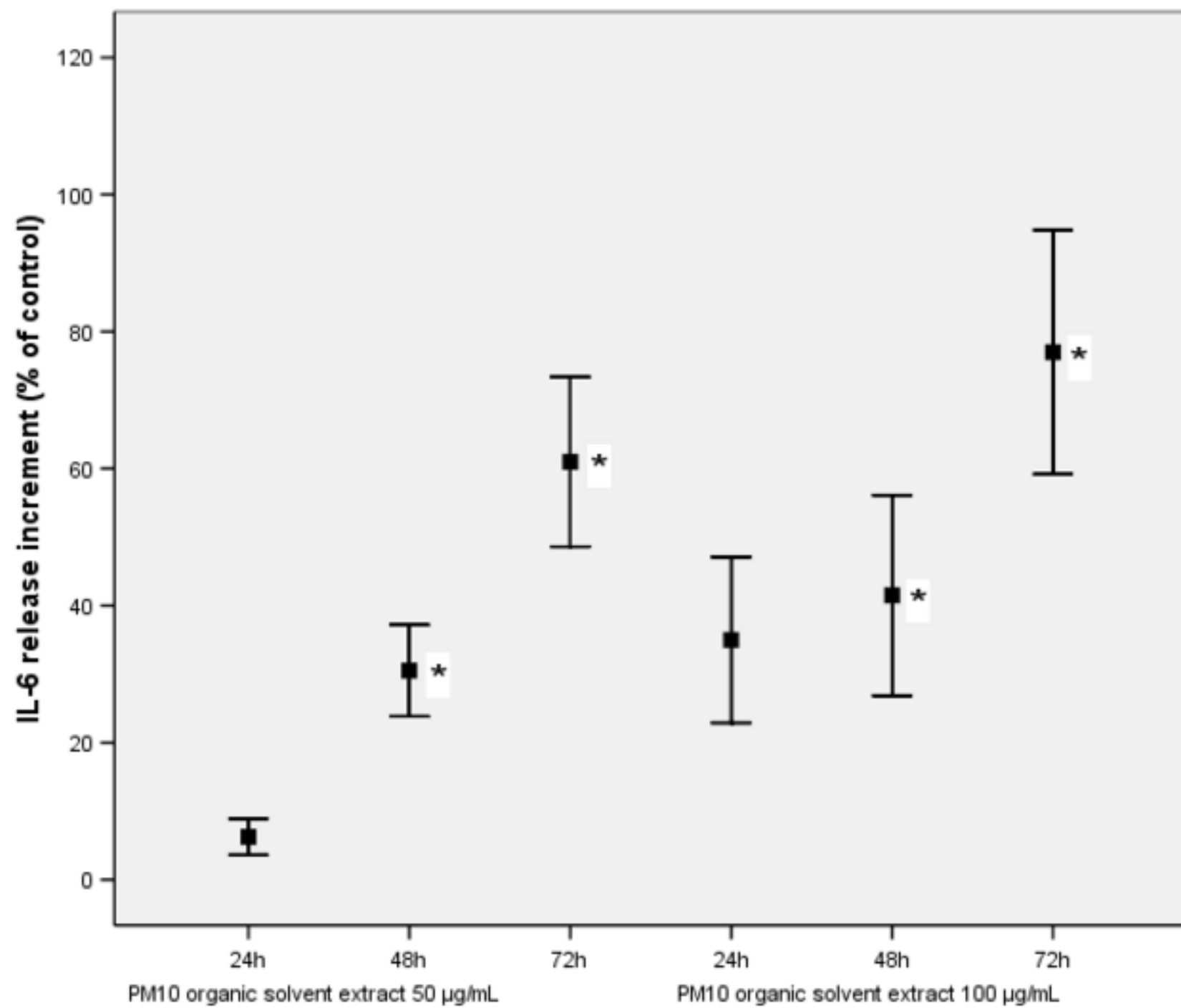


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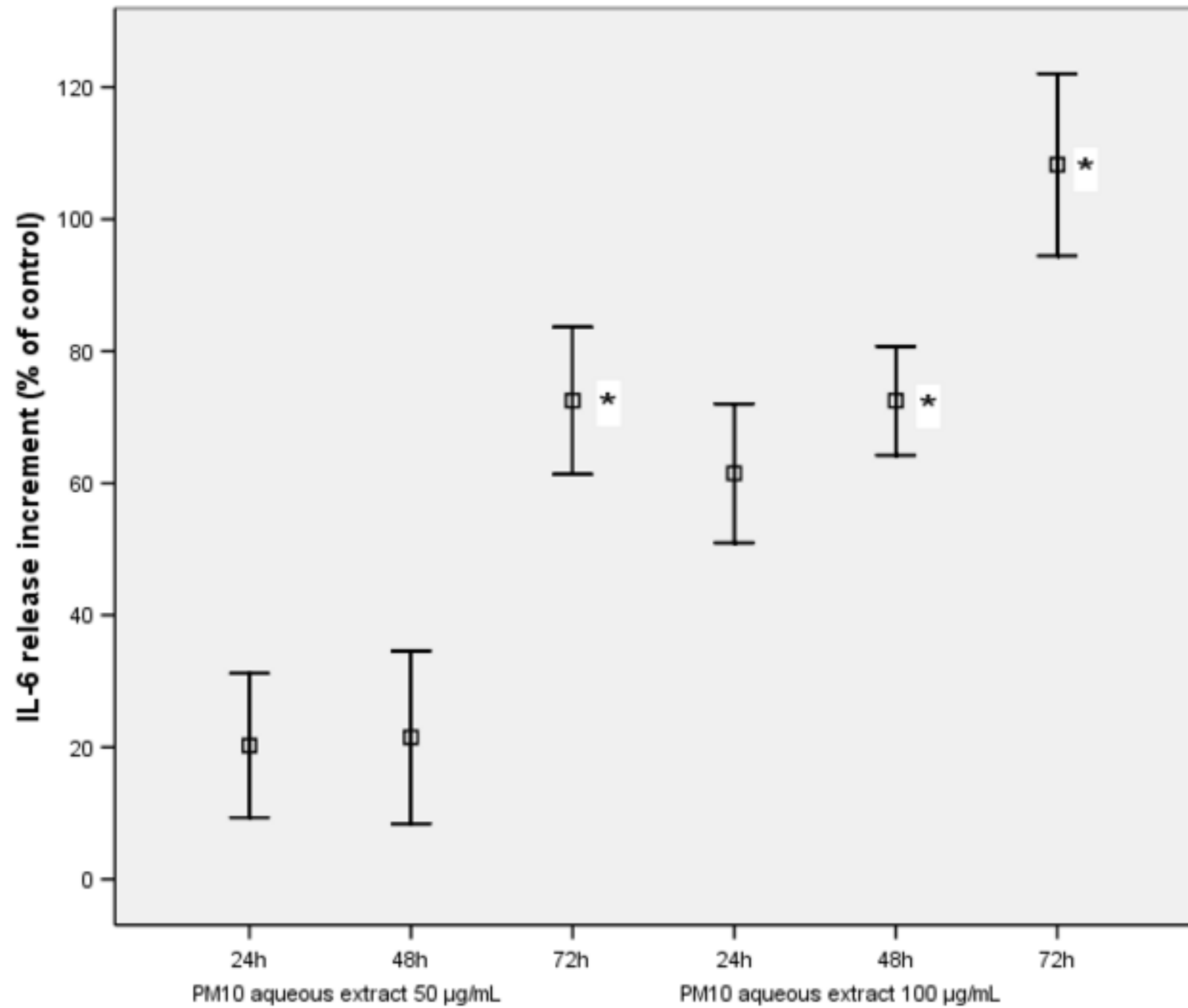


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