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**Organic Honey Supplementation Reverses Pesticide-Induced Genotoxicity by Modulating DNA Damage Response**

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Keywords:	Organic Honey, mitochondria, DNA damage response, Polyphenols, Pesticides

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3 **Organic Honey Supplementation Reverses Pesticide-Induced Genotoxicity by Modulating**  
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5 **DNA Damage Response**  
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36 **Abbreviations:** **BER**, base excision repair; **CPF**, chloryrifos; **DCFDA**, dichlorofluorescein  
37 diacetate; **DDR**, DNA damage response; **DTT**, Dithiothreitol; **ENDO III**, endonuclease III; **ESI**,  
38 electrospray ionization; **ETC**, electron transport chain; **FPG**, formamido pyrimidine glycosylase;  
39  
40 **GLY**, Glyphosate; **Mn-SOD**, Mn-superoxide dismutase; **mtROS**, mitochondrial ROS; **NAC**, N-  
41 acethyl cysteine; **NRF2**, Nuclear factor erythroid 2-related factor 2; **OP**, organophosphorus;  
42  
43 **ORAC**, Oxygen radical absorbance capacity; **OGG1**, 8-oxoguanine DNA glycosylase 1; **PBS**,  
44 phosphate-buffered saline; **ROS**, Reactive oxygen species; **TCP**, 3,5,6-trichlor-2-piridinol.  
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52 **Keywords:** Organic Honey, Mitochondria, DNA damage response, Polyphenols, Pesticides  
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**Abstract**

**Scope:** Glyphosate (GLY), and organophosphorus (OP) insecticides such as chlorpyrifos (CPF) may cause DNA damage and cancer in exposed individuals through mitochondrial dysfunction.

Polyphenols ubiquitously present in fruits and vegetables, have been viewed as antioxidant molecules, but also influence mitochondrial homeostasis. Here, honey containing polyphenol compounds was evaluated for its potential protective effect on pesticide-induced genotoxicity.

**Methods and results:** Honey extracts from four floral organic sources were evaluated for their polyphenol content, antioxidant activity and potential protective effects on pesticide-related mitochondrial destabilization, ROS formation and DNA damage response in human bronchial epithelial and neuronal cells. The protective effect of honey was, then evaluated in a residential population chronically exposed to pesticides. The four honey types showed a different polyphenol profile associated with a different antioxidant power. The pesticide-induced mitochondrial dysfunction parallel ROS formation from mitochondria (mtROS) and consequent DNA damage. Honey extracts efficiently inhibited pesticide-induced mtROS formation, and reduced DNA damage by upregulation of DNA repair through NFR2. Honey supplementation enhanced DNA repair activity in a residential population chronically exposed to pesticides, which resulted in a markedly reduction of pesticide-induced DNA lesions.

**Conclusion:** These results provide new insight regarding the effect of honey containing polyphenols on pesticide-induced DNA damage response.

## 1 Introduction

Human health is determined by various interacting factors, among which lifestyle, environment and diet play an important synergic role in the prevention of several disease, including cancers. Human intervention trials have provided evidence for protective effects of various (poly)phenol-rich foods against chronic disease, including cardiovascular disease, neurodegeneration, and cancer [1-4].

While there are considerable data suggesting the benefits of (poly)phenol rich-diet, conclusions regarding their preventive potential remain unresolved due to several limitations in existing studies.

Several polyphenols have been shown to effectively modulate pathways that define mitochondrial biogenesis, mitochondrial membrane potential (i.e., mitochondrial permeability transition pore opening and uncoupling effects), mitochondrial electron transport chain (ETC) and ATP synthesis [5]. Polyphenols can acts as antioxidants either through a ROS-scavenging mechanism or actions promoted at the ROS-removing level by directly inhibiting the major ROS-forming enzymes.

Recent evidence indicates that, by acting at the ETC-complexes level, certain polyphenols would be able to modulate the rate of mitochondrial superoxide production [6]. The pathogenesis of both neurological disorders and cancer has been related to mitochondrial dysfunction [7, 8].

Mitochondrial dysfunction and consequent energy depletion are the major causes of oxidative stress resulting to bring alterations in the ionic homeostasis causing loss of cellular integrity.

Among various environmental toxicants, pesticides have been shown to affect mitochondria. Exposure to chlorpyrifos (CPF), an insecticide widely used in agriculture, was found to decrease the activity level of Krebs cycle enzymes and ETC protein complexes [9, 10]. As well, Glyphosate (GLY), the active ingredient in Roundup®, has also been shown to severely deplete Manganese (Mn) levels, associated with Mn-superoxide dismutase (Mn-SOD) suppression, and mitochondrial dysfunction [11].

In this context, honey is a good source of physiologically active natural compounds. Here, honey extracts from four floral organic sources (acacia, chestnut, orange tree, woodland) were

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2  
3 evaluated for their polyphenol content, antioxidant activity and potential protective effects on  
4  
5 pesticide-related mitochondrial destabilization, ROS formation and DNA damage response (DDR)  
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7 in human bronchial epithelial cells (pesticide penetration way) and neuronal cells (pesticide target  
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9 cells). The protective effect of honey was, also *in vivo* evaluated in a population chronically  
10  
11 exposed to pesticides.  
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## 14 15 16 17 18 **2 Materials and methods**

### 19 20 21 **2.1 Sample extraction**

22  
23 Phenolic compounds for HPLC analysis were extracted from honey (acacia, Ext-1; chestnut, Ext-2;  
24  
25 orange tree, Ext-3; woodland, Ext-4) as described previously [12]. About 3 g of honey was  
26  
27 dissolved in 15 ml of acidified water (pH=2 with HCl), stirring at room temperature until  
28  
29 completely fluid. The solution was mixed with 4 g Amberlite XAD-2 (pore size 9 nm, particle size  
30  
31 0.3–1.2 mm, Sigma) and stirred for 10 min. The Amberlite particles were then packed in a glass  
32  
33 column (30 cm x 3 cm), washed with acidified water (pH=2 with HCl, 10 ml) and subsequently  
34  
35 rinsed with distilled water (30 ml). The phenolic compounds remained adsorbed on the column,  
36  
37 while sugars and other polar compounds eluted with the aqueous solvent. The whole phenolic  
38  
39 fraction was then eluted with methanol (3 ml) and taken to dryness by vacuum Concentrators  
40  
41 (SpeedVac,- Thermo Scientific) . The residue was re-suspended in distilled water to a concentration  
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43 of 100 mg/ml.  
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### 50 51 **2.2 HPLC–DAD–MS analysis**

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53 All HPLC analyses were performed using an HP 1100 Series Instrument (Hewlett–Packard,  
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55 Wilmington, DE) equipped with a binary pump (model G1312A) delivery system, a degasser  
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57 (model G1322A), an autosampler (Automatic Liquid Sampler, ALS, model G1313A), a HP diode-  
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array UV–VIS detector (DAD, model G1315A) and a HP-single-quadrupole mass spectrometer detector (MS, model G1946A). Separations were carried out on a reverse phase column Eclipse XDB-C18, 5  $\mu\text{m}$  250, 3.0 mm ID, (Agilent Technologies, Santa Clara, CA, USA) with a Securityguard precolumn filter. All solvents were HPLC-grade and filtered with a 0.45  $\mu\text{m}$  filter disc. Elution was carried out with a 1% acetic acid (solvent A) and acetonitrile (solvent B) and with a linear gradient. All the analyses were carried out at room temperature, with an injected volume of 10  $\mu\text{l}$  and a flow rate of 0.5 ml/min. UV spectra were recorded from 210 to 600 nm, whereas the chromatograms were monitored at 280 and 330 nm, since the majority of the honey flavonoids and phenolic acids show their UV absorption maxima around these two wavelengths. MS analyses, performed with the same HPLC–DAD condition, were carried out using an electrospray ionization (ESI) interface in positive and negative mode at the following conditions: drying gas flow ( $\text{N}_2$ ), 11.0 L/min; nebulizer pressure, 60 psi; gas drying temperature, 350°C; capillary voltage, 4000 V; fragmentor voltage, 90 V; scan range, m/z 120–850. Ionisation of the phenolic compounds in negative mode gave high sensitivity and selectivity and ionisation in positive mode provided extra certainty in the determination of the molecular masses. The honey phenolic acids and flavonoids were identified by chromatographic comparison with reference standards and by matching their UV spectra with those of the markers. The individual phenolic compounds were quantified by their absorbance in the HPLC chromatograms against external standards, using ferulic acid at 280 nm for phenolic acids and chrysin at 330 and 280 nm for flavonoids. Integration and data elaboration were performed using HP ChemStation software (Hewlett–Packard, Palo Alto, CA). The quantitative analyses were carried out in triplicate and expressed as micrograms per 100 g of honey.

### 2.3 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed by OxiSelect™ ORAC Activity Assay Kit (Cell Biolabs) according to the manufacturer's instructions. Dilutions of samples and Trolox (reference antioxidant, water-soluble vitamin E analogue) were incubated in 96-well plates for 10 min at 37 °C

1  
2  
3 with fluorescein. After this period, AAPH was added to the mixture. Fluorescence was read ( $\lambda_{exc}$   
4 485 nm and  $\lambda_{em}$  520 nm) every 20 min for a total of 160 min using a fluorescence plate reader  
5  
6 (Infinite F200 PRO, Tecan). The area under the curve (AUC) was calculated for each sample and  
7  
8 compared with that of Trolox. ORAC values are expressed as mmol Trolox equivalent.  
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## 11 12 13 **2.4 Cell culture and treatment**

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15 Human bronchial epithelial cells (BEAS-2B) and neuronal SHSY-5Y cell line were used as a model  
16  
17 of in vitro evaluation of mutagenesis and neurodegenerative disease studies, respectively. BEAS-2B  
18  
19 (ATCC® CRL9609™), and SHSY-5Y cells (ATCC® CRL2266™) were grown in in the DMEM  
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21 medium with antibiotics and 10% FBS, and regularly checked for absence of mycoplasma  
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23 contamination using the PCR Mycoplasma Test. Cells were treated with pesticides (ranging 10-  
24  
25 1000  $\mu$ M) in absence or presence of honey-extracts (5  $\mu$ g/ml) added 2h before pesticide treatments.  
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28 Chronic exposure to pesticides was established by incubating BEAS-2B and SHSY-5Y cells with  
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30 GLY and CPF (100  $\mu$ M in fresh medium, three times a week) over a period of 6 months.  
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## 34 35 **2.5 Cell proliferation assay and mitochondrial activity**

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37 Cells were seeded at  $10^4$  cells/well in a 96-well, allowed to attach overnight, and over time (24-48-  
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39 72 h) treated with increasing concentrations of pesticides (0, 10, 100, 500, 1000  $\mu$ M). After  
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41 treatment, 50  $\mu$ l of crystal violet (2% crystal violet in 2% ethanol) were added, and incubated for 5  
42  
43 min. After four washing, 200  $\mu$ l of isopropanol was added to dissolve the crystals, and absorbance  
44  
45 read at 570 nm in an ELISA plate reader (Sunrise, Tecan, Männedorf, Swiss), and the results  
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47 expressed as rate of proliferation (abs/time).  
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50  
51 For mitochondrial activity, the resazurin assay was performed. Cells were incubated with  
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53 resazurin (6  $\mu$ M) in the presence and absence of pesticides (0, 10, 100, 500, 1000  $\mu$ M).  
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56  
57 Fluorescence intensity was read at 0-240 min in a fluorescence plate reader (Infinite F200 PRO,  
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59 Sunrise, Tecan, Männedorf, Swiss). The excitation and emission filters were set at 485 nm and 530  
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1  
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3 nm, respectively. The results were normalized to the total protein using the Bradford assay (Sigma),  
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5 and expressed as rate (MFI/min).  
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## 8 9 **2.6 Assessment of ROS**

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11 Intracellular reactive oxygen species (ROS) levels were estimated using the fluorescent dye 2'7'-  
12 dichlorofluorescein diacetate (DCFDA; oxidized by hydrogen peroxide to DCF). BEAS-2B and  
13 SHSY-5Y cells ( $2 \times 10^5$ ) were seeded in 6-well plates, supplemented with 20  $\mu$ M DCFDA per well,  
14 and treated with pesticides (10  $\mu$ M and 100  $\mu$ M) in presence or absence of honey-extracts (5  
15  $\mu$ g/ml). After treatment the fluorescent probe was removed, the cells washed and re-suspended in  
16 PBS, and analyzed by flow cytometry (FACS Calibur, Becton Dickinson). The level of ROS was  
17 expressed fold increase in fluorescence respect to control (untreated cells).  
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## 28 29 **2.7 DNA damage**

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31 DNA breaks and oxidized purine and pyrimidine bases were measured using a Comet assay  
32 described elsewhere [13]. Briefly, lymphocytes or treated and control cells, were embedded in  
33 agarose on a microscope slide, lysed with Triton X-100 and 2.5 M NaCl to produce nucleoids and  
34 treated with 0.3 M NaOH/1 mM EDTA before electrophoresis in this solution. Oxidized bases were  
35 detected by including an extra step, in which nucleoids in the gel are digested with a repair  
36 endonuclease specific for oxidized pyrimidines (endonuclease III, ENDO III) or recognizing altered  
37 purines, including 8-oxodG (FPG protein). Slides were incubated for 30 min with 50  $\mu$ l of either  
38 buffer, FPG and ENDO III (generously gift from Prof. Andrew Collins, University of Oslo, Oslo,  
39 Norway). DNA single strand breaks (SSBs), with or without enzymatic treatment, were estimated as  
40 arbitrary units (au). Oxidized purine and pyrimidine bases were calculated by subtracting the value  
41 without enzyme incubation (i.e. SSBs) from the value with enzyme incubation. The extent of DNA  
42 migration was evaluated by visual scoring by an independent observer.  
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## 2.8 DNA repair assay

The activity of DNA repair was evaluated as capacity of cell extract to repair the oxidized purine (8-oxodG) introducing DNA breaks detected as previously described [13,14]. Briefly, cell extract was prepared from  $2.0 \times 10^6$  cells in extract buffer (45 mM Hepes, 0.4 M KCl, 1.0 mM EDTA, 1mM DTT, and 10% (v/v) glycerol adjusted to pH 7.8 with KOH), and was cryopreserved at  $-80^\circ\text{C}$  until used in the DNA repair assay. Lysate (50  $\mu\text{l}$ ) was supplemented with 12  $\mu\text{L}$  of extract buffer containing 1% Triton X-100 and was then centrifuged for 5 min at  $4^\circ\text{C}$  at 14000 x g. The supernatant was diluted with four volumes of reaction buffer with 0.25 mM EDTA, 2% glycerol, and 0.3 mg/ml BSA, pH 7.8. The protein extract (50  $\mu\text{g}$ ) was added over a slide with a substrate consisting of nucleoid DNA with oxidized purine bases from A549 cells previously exposed to Ro 19-8022 (generously gift from Hoffmann-LaRoche, Basel, Switzerland) plus light, which specifically induces 8-oxodG formation. The slides were incubated for 45 min at  $37^\circ\text{C}$  and comet assay (alkaline unwinding, electrophoresis, neutralization, staining, and evaluation) was carried out as described above. The time-course of break production on the specifically damaged DNA substrate is a measure of repair capability. The ability of cells to repair was also evaluated by measuring residual DNA damage over a period of incubation. Briefly, cells were seeded in a 24-well plate, and damage to DNA was introduced by incubating them with 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in PBS for 5 min on ice ( $T_b$ ). After treatment, the PBS was replaced with RPMI-1640 medium containing 10% foetal bovine serum and incubated at  $37^\circ\text{C}$  for 6 h. At regular interval times ( $T_n = 30, 60, 120$  min), the cells were collected and SSBs, FPG-sites, ENDO III-sites evaluated by comet assay. Residual damage (%) at different time points was determined as follows:  $100 \times [\text{DNA damage at time T after treatment } (T_n) - \text{DNA damage of untreated cells } (T_0) / \text{DNA damage immediately after treatment } (T_b) - \text{DNA damage of untreated cells } (T_0)]$ . The  $t_{1/2}$  (h) were determined using plots of data obtained from the initial time ( $t_i$ ) of induced DNA damage level ( $C_i$ ) to the final time ( $t_f$ ) when damage was completely repaired ( $C_f$ ), according to the following formulas:  $K = (\log C_i - \log C_f) / (t_f - t_i)$ ,  $t_{1/2} = \log 2 / K$ .

## 2.9 Western blot analysis

Cells ( $3 \times 10^5$  per well in 6-well plates) were harvested, and the pellet re-suspended in the cytoplasm extract (CE) buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP40, 1mM DTT and 1 mM PMSF, adjusted to pH 7.6). After 5 min incubation on ice, the pellet was centrifuged at 1500 rpms for 5 min, and the supernatant containing the cytosolic fraction obtained. The remaining pellet was washed in CE buffer, and lysed in the nuclear extract (NE) buffer (20 mM Tris-HCl, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1 mM PMSF and 25% (v/v) glycerol, adjusted to pH 8.0). After 20 min incubation on ice, the pellet was centrifuged at 12000 rpms for 10 min at 4°C, and the supernatant was collected. The nuclear extracts were stored at -80°C until used. For western blot analysis, protein samples (50 µg per lane) were resolved using 4-12% SDS-PAGE (Life Technologies), and transferred to nitrocellulose membranes, and incubated overnight with anti-NRF2 (Cell Signaling Technology, Danvers, MA) and anti-OGG1 (Origene, Rockville, MD). Lamin (Bethyl, Montgomery, TX, USA) was used as loading control. After incubation with an HRP-conjugated secondary IgG (Sigma), the blots were developed using the ECL detection system (Pierce Biotechnology, Rockford, IL, USA). Band intensities were visualized by ChemiDoc using the Quantity One software (BioRad, Hercules, CA).

## 2.10 Ethics statement

All subjects filled a questionnaire including their informed consent. The study was carried out according to the Helsinki Declaration and the samples were processed under approval of the written consent statement of the Ethical Committee A.O.U. “Ospedali Riuniti” of Ancona, Italy (n° 211584), according to Ministerial Decree (DM 12/05/2006).

## 2.11 Recruitment of Study Participants

Residential population exposed to pesticides was recruited from October 2015 to June 2015 at the Medical Centre of Tuenno (TR), Italy. The enrolled subjects were residents in the Val di Non

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2  
3 (Trento, Italy), an area at intensive culture of apple orchards. In this area, from March to middle of  
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5 October apple orchards undergo to series of pesticide treatments as spray solution preparation,  
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7 which includes fungicides (boscalid, captan, fluazinam, iprodione, penconazole), herbicides  
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9 (glyphosate), acaridae (Bromopropylate), and insecticides (chlorpirifos etile, malathion, piretrine,  
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11 metossifenozone). Therefore, more than 30 treatments, often as mix of different substances, are used  
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13 within the year, with major exposure at the May-June period. Due to the conformation of the area,  
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15 which is constituted by valley closed to mountains, the pesticides remain in the environment  
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17 affecting also the residential population. Residents chronically exposed to pesticides were 34  
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19 subjects, who consented to participate in the study, and included teachers and employees, engineers,  
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21 architected, all subjected to passive exposure to pesticides. The participants were interviewed by  
22  
23 trained personnel and answered a detailed questionnaire that included life-style including dietary  
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25 consumption, smoking, environmental and occupational exposure.  
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29  
30 The control group consisted of healthy subjects (n=40), having no previous or current  
31  
32 occupational/environmental exposure to pesticides, recruited from November 2015 to June 2015.  
33  
34 The subjects were undergoing medical check-up at the Clinic of Occupational Medicine of the  
35  
36 University Hospital of Ancona, Italy. None of them had ever been exposed to pesticides as  
37  
38 documented by their environmental and occupational histories.  
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### 43 **2.12 Sample collection**

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45 Three periods of exposure to pesticides were surveyed: no pesticides-exposure, when the pesticides  
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47 were not used (P0, February-March period), low-exposure, when pesticides were used occasionally  
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49 (P1, October-November period), and high exposure, in which pesticides were used regularly on a  
50  
51 weekly basis (P2, May-June period). Blood samples were collected in fasting subjects at P0 and at  
52  
53 the P1 and P2 periods, before (S0) and after two weeks of organic honey dietary intake (50 mg/day,  
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55 S1) administered in substitution of other sweeteners. All participants provided for blood sample for  
56  
57 each period at the time of interview. Blood samples were collected into EDTA tubes and used for  
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3 lymphocyte isolation as previously described [13,14]. A morning urine samples (~50 ml) were  
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5 collected in polypropylene containers (Starplex Scientific, Canada) at all three exposure periods  
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7 (P0, P1, and P2), and stored frozen at -80°C until analysis. The control subjects provided for urine  
8  
9 samples at the moment of enrollment.  
10

### 11 12 13 14 **2.13 Air sample collection**

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16 Air sampling was conducted for 24h (9.00 AM to 9.00 AM of the day after) in three areas at the  
17  
18 high-exposure period (P2). Air samples were collected near the apple orchards by using sampling  
19  
20 pumps connected to the sampler (Solid sorbent tube; OVS-2 tube: 13 mm quartz; XAD-2, 270  
21  
22 mg/140mg). The pump was set at a flow rate of 2 L/min and the sampler was set to connect along  
23  
24 the calibrated sampling pump with tube. Pump flow-calibration checks were performed before and  
25  
26 at the end of the sampling period. At the completion of sampling, the sampler was capped at both  
27  
28 ends with plastic caps and packed for shipment. After collection, the samples were labeled and  
29  
30 frozen in an icebox to be sent to the laboratory. Samplers were used and the solvent was extracted  
31  
32 and analyzed by GC-ICP-MS (Agilent 7000C GC Triple Quadrupole GC/MS system).  
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### 38 39 **2.14 3,5,6-trichlor-2-piridinol analysis**

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41 Biological monitoring of exposure was performed by determining chlorpirifos ethyl metabolite  
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43 (3,5,6-trichlor-2-piridinol, TCP) in urine samples according to the method previously described  
44  
45 [15]. The results were expressed as a function of creatinine (creat) levels (mg/g creat).  
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### 49 50 **2.15 Statistical analysis**

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52 Results were expressed as mean  $\pm$  S.D., or median (25° percentile-75° percentile). Comparisons  
53  
54 among groups of data were made using one-way analysis of variance (ANOVA) with Tukey post-  
55  
56 hoc analysis. The two-tailed Student's t-test was used to compare two groups. ANOVA repeated  
57  
58 measure with Sidak post-hoc test was used to evaluate differences among the P0, P1-S0, P1-S1,  
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3 P2-S0, and P2-S1 time points. The data were analyzed by the Statistical Package Social Sciences  
4  
5 (version 19) software (SPSS, Chicago, IL, USA) and p-values less than 0.05 were considered  
6  
7 significant.  
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### 10 11 **3 Results**

#### 12 13 **3.1 Polyphenol profile and antioxidant capacity of honey extracts**

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17 All the tested organic honey samples had similar but quantitatively different phenolic profiles and  
18  
19 up to 20 peaks could be assigned to phenolic compounds and identified as phenolic acids (peaks 1–  
20  
21 10), abscisic acid (peaks 11 and 12) and flavonoids (peaks 13–20) (*Supplementary Figure S1*). As  
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23 reported in Figure-1A, the Ext-4 was richer in polyphenols respect to other extracts (Ext-1, Ext-2  
24  
25 and Ext-3), showing high levels of caffeic acid, coumaric acid, Ferulic acid, iso-Ferulic acid,  
26  
27 pinobanksin and pinocembrin. The higher content of polyphenols reflected higher antioxidant  
28  
29 capacity followed by Ext-4, Ext-2, Ext-3 and Ext-1 (Figure-1B).  
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#### 37 38 **3.2 Honey extracts attenuate mitochondrial destabilization induced by pesticides**

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40 BEAS-2B and SHSY-5Y cell lines were treated with increased concentration of GLY and CPF  
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42 ranging 10-1000  $\mu\text{M}$ , and cell proliferation, mitochondrial activity evaluated. GLY induced cell  
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44 proliferation in a dose-dependent manner, associated with increased mitochondrial activity at low  
45  
46 concentration, which was reduced at increased doses ( $>100 \mu\text{M}$ ) in BEAS-2B cells (Figure 2A). No  
47  
48 effect of GLY was found in SHSY-5Y cells (Figure B). Conversely, CPF inhibited cell proliferation  
49  
50 in both cell lines, while inducing mitochondrial activity at concentrations below 100  $\mu\text{M}$ . Therefore,  
51  
52 two sub-toxic doses of GLY and CPF, 10  $\mu\text{M}$  (Low-dose) and 100  $\mu\text{M}$  (High-dose) were used to  
53  
54 establish a genotoxic stimulus model. As reported in Figure 2C, both pesticides at low-dose, and in  
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56 a major extent at high-dose induced ROS formation measured in terms of fluorescence by DCF. To  
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3 investigate the effect of honey extracts, BEAS-2B and SHSY-5Y cells were treated with GLY and  
4 CPF (10  $\mu$ M and 100  $\mu$ M) over time in presence or absence of extracts (5  $\mu$ g/ml) and mitochondrial  
5 activity and ROS formation evaluated. The increased mitochondrial activity and ROS formation  
6  
7 induced by pesticides (GLY and CPF), peaking at 1-3 h and 24 h of pesticide exposure  
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11 (*Supplementary figure S2A,B*), were reversed by honey-extracts in both cell lines (Figure 3A,B, and  
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13 *Supplementary Figure S3A,B*).

### 14 15 16 17 18 19 20 **3.3 Honey extracts inhibit pesticides-induced DNA damage**

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23 Mitochondria are the major producer of intracellular ROS, and have been linked to the cause of  
24 aging and other chronic diseases [16]. To determine a direct relationship between intracellular ROS  
25 levels and pesticides-induced mitochondrial destabilization, the cells were pretreated with the  
26  
27 superoxide scavenger Tiron (10 mM), ROS scavenger N-acethyl cysteine (NAC, 10 mM), honey-  
28  
29 extract (5  $\mu$ g/ml), and ROS formation was induced by GLY and CPF treatments (10  $\mu$ M and 100  
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31  $\mu$ M) for 24h. As reported in figure 4A, the ROS induced by pesticides were inhibited by Tiron, and  
32  
33 in a major extent by NAC and honey-extract. The inhibition of ROS by honey-extract paralleled the  
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35 reduction of pesticides-induced DNA damage evaluated as single strand breaks (SSBs) and oxidized  
36  
37 DNA base accumulation (FPG-sites and ENDOIII-sites) both in BEAS-2B cells (Figure 4B, C) and  
38  
39 SHSY-5Y cells (Figure 4D). Thus, suggesting that pesticides affect mitochondria homeostasis by  
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41 inducing ROS production (mtROS), which in turn induce DNA damage. This process was  
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43 significantly inhibited by honey extracts.  
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### 53 54 **3.4 Honey extracts rescue cells to pesticides-induced DNA repair inhibition**

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56 Endogenous DNA lesions are the results of a balance between DNA damage and DNA repair  
57  
58 (steady state). The failure to detect and accurately repair these lesions can give rise to cells with  
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3 high levels of endogenous DNA damage, deleterious mutations, or genomic aberrations. Such  
4  
5 genomic instability can lead to the activation of specific signaling pathways, including the DNA  
6  
7 damage response (DDR). Incubation of cells with honey-extracts (5 µg/ml) enhanced DNA repair  
8  
9 activity in BEAS-2B and slightly in SHSY-5Y cells (Figure 5A). As reported in Figure 5B, the cell  
10  
11 showing higher DNA repair activity respect to their non-supplemented counterparts resulted in an  
12  
13 increased capacity to repair DNA damage induced by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Conversely, both  
14  
15 GLY and CPF incubation (100 µM for 24h) inhibited cellular DNA repair activity, which was  
16  
17 reversed by honey-extract (Figure 5C). In addition, chronic exposure to pesticides was established  
18  
19 by incubating BEAS-2B and SHSY-5Y cells with GLY and CPF (100 µM in fresh medium, three  
20  
21 times a week) over a period of 6 months. Cells chronically exposed to pesticides showed lower  
22  
23 DNA repair activity respect to untreated cells (Controls). The incubation of honey-extract increased  
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25 DNA repair activity at levels similar to control cells (Figure 5D).  
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### 33 **3.5 NRF2 plays an important role in the modulation of the DNA repair activity**

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36 Nuclear factor erythroid 2-related factor 2 (NRF2) is a known regulator of the antioxidant response  
37  
38 [17]. NRF2-mediated regulation of protective enzymes like oxidative DNA damage repair gene 8-  
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40 oxoguanine DNA glycosylase 1 (OGG1) [18]. OGG1 plays an essential role in the removal of 8-  
41  
42 oxo-guanines (FPG-sites) from nuclei. We examined the nuclear translocation of NRF2 and OGG1  
43  
44 in BEAS-2B and SHSY-5Y cells treated with GLY and CPF (100 µM, 24 h) with and without  
45  
46 honey extract (5 µg/ml), and compared it with NRF2-OGG1 expression in their control cells.  
47  
48 Pesticide incubation induces nuclear translocation of NRF2, which paralleled increased OGG1  
49  
50 nuclear expression (Figure 6A,B). Honey extract from woodland (Ext-4) alone or in combination  
51  
52 with pesticides significantly increased nuclear protein expression of NRF2 and OGG1 (Figure  
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54  
55 6A,B). Notably, the SHSY-5Y cells were less reactive to pesticide- and honey extract-induced  
56  
57 NRF2/OGG1 nuclear translocation.  
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### 3.6 Honey-supplementation enhances DNA damage response (DDR) in residents chronically exposed to pesticides

The effect of honey containing polyphenols on DDR was *in vivo* evaluated in a population chronically exposed to pesticides. Residents from an area at intensive use of pesticides (Val di Non, Trento, Italy), and subjects living in a pesticide-free area, negative for urine TCP, were included in the study. The residents were active, mainly consumed fish and organic foods, with low consumption of meat and sweets (Supplementary Figure S4). The demographic and anthropometric characteristics are shown in Table 1A. Based on the calendar of treatments the DDR was evaluated in the population at three periods: P0, no pesticides-exposure; P1, low-pesticide exposure, and P2, high-pesticide exposure. At the P1 and P2 periods, the DDR was evaluated in subjects before and after 2-weeks honey supplementation (50 gr/Die). All the residents were positive for the urine metabolite TCP in all periods analyzed, showing high TCP levels at high-exposure period (Table 1B). Among the multi-residual pesticides analyzed (see list of compounds in Supplementary Figure S5), only chlorpyrifos ethyl, Difenconazole, Fluazinam, and methoxyfenozide have been detected in air samples (Table 1B). In the period of no-exposure, the population chronically exposed to pesticides showed reduced DNA repair activity associated with a defective DNA damage recovery respect to control subjects (Figure 7). Although DNA repair was low in these subjects, they showed comparable levels of FPG and ENDOIII lesions respect to controls (Figure 7). The pesticide exposure further reduced the DNA repair activity, which was associated with increased FPG and ENDOIII sites formation. Notably, the honey supplementation enhanced DNA repair activity, thus resulting in a markedly reduction of FPG and ENDOIII lesion accumulation.

#### 4 Discussion

Honey is a polyphenol-rich food, which has been shown to exhibit several biological activities, including antimicrobial [19], anti-inflammatory [20], antioxidant [21], cardio-protective properties [22] and anticancer [23]. Compelling evidence has shown that the polyphenol-enriched fraction from honey can suppress cancer in vivo model [24, 25]. To date, approximately 300 varieties of honey have been identified, which are characterized by a different mixture of approximately 30 different polyphenols [26,27]. The chemical composition contributes to honey bioactivity. In the present study we evaluated the extracts from four organic honey varieties (acacia, chestnut, orange tree, woodland) for their polyphenol composition, antioxidant effects and effect on toxicity induced by pesticides. Even though the 4-honey extracts had different antioxidant activity, reflecting the different polyphenol content and profile (*cf* Fig.1), they showed comparable effect on cell genotoxicity induced by pesticide exposure.

Pesticides are compounds highly used in conventional agriculture practice, and multi residue of pesticides was found in non-organic fruit, vegetable and honeys [28,29]. Environmental, occupational and food exposure have been associated with health risk [30-33]. During occupational and residential exposure, pesticides are prevalently absorbed through the airways, and the neurons are one of target of these compounds. Prospective cohort studies have shown that prenatal or early childhood exposure of CPF causes adverse neurodevelopmental outcomes, such as decreased head circumference, poorer intellectual development and cognitive deficits, mental and motor delays, as well as neurodegenerative diseases [34,35]. Meta-analyses showed that phenoxy herbicides, carbamate insecticides, organophosphorus insecticides and the active ingredient lindane, an organochlorine insecticide, were positively associated with non-Hodgkin lymphoma; B cell lymphoma was positively associated with phenoxy herbicides and the herbicide glyphosate [36]. Here, bronchial BEAS-2B cell (airway cells) and neuronal SHSY-5Y cell line (target cells) were used as a model to evaluate in vitro pesticide exposure. The two cell lines differently responded to

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2 pesticides (GLY and CPF). GLY induced cell proliferation in BEAS-2B cells, even at low dose, but  
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4 not in SHSY-5Y cells. Conversely, CPF decreased cell proliferation in a dose-dependent manner in  
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6 both cell lines. Mitochondrial damage has been implicated to play a key role in pesticide-induced  
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8 neurotoxicity [37,38]. We showed that GLY and CPF treatment caused increased mitochondrial  
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10 activity at low doses, while inhibiting mitochondrial activity at higher concentrations. The altered  
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12 mitochondrial dysfunction was associated with ROS generation in both cell lines (*cf* Fig.2). In  
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14 particular, superoxide production by the mitochondrial respiratory chain can increase dramatically  
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16 when the mitochondrial membrane potential is high (high mitochondrial activity) associated with  
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18 low ATP production [39]. Pesticides mainly induced ROS through mitochondrial destabilization,  
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20 most of ROS were reduced by the superoxide scavenger Tiron. The mtROS produced paralleled  
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22 DNA damage in both cell lines (*cf* Fig.4). The honey extracts markedly restored the mitochondrial  
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24 activity, and inhibited ROS-induced DNA damage (*cf* Fig.3-4).  
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31 Genome instability is a prerequisite for the development of degenerative and cancer  
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33 diseases. It occurs when genome maintenance systems fail to safeguard the genome's integrity as  
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35 consequence of inherited defects, or induced via exposure to environmental agents [40]. Various  
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37 DNA repair pathways prevent the persistence of such DNA lesions, maintaining genome integrity.  
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39 Alterations to the epigenome may also lead to genome instability in an indirect way. Epigenetic  
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41 changes can affect DNA repair efficiency and fidelity by changing the expression of DNA repair  
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43 genes [41,42]. In the last decade, a number of studies have shown that nutrients can affect metabolic  
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45 traits by altering the structure of chromatin and directly regulate both transcription and translational  
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47 processes [43]. In this context, dietary polyphenol-targeted epigenetics becomes an attractive  
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49 approach for disease prevention and intervention [44]. Here, we found that honey-polyphenols  
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51 modulate the activity of DNA repair involved in the DNA base excision repair (BER) system.  
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53 Honey extracts by themselves increased DNA repair activity, associated with an enhanced kinetic to  
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55 repair the damaged DNA in BEAS-2B cells. In this context, honey extracts reversed the reduced  
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3 DNA repair activity induced by pesticide exposure. This phenomenon was not observed in neuronal  
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5 cells, even though the honey extracts prevented the down-regulation of DNA repair activity  
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7 mediated by pesticides in these cells (*cf* Fig.5).  
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10 The DNA glycosylase OGG1 is the main BER enzyme that repairs 8-oxoG, which is a  
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12 critical mutagenic lesion. The level of OGG1 expression may be modulated by a variety of stimuli  
13  
14 including oxidative stress [45]. The transcription factor NRF2 has emerged as a master regulator of  
15  
16 intracellular redox homeostasis by controlling the expression of a battery of redox-balancing  
17  
18 antioxidants and phase II detoxification enzymes. Nrf2 activation has been shown to mitigate a  
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20 number of pathologic mechanisms associated with Alzheimer's disease, Parkinson's disease,  
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22 amyotrophic lateral sclerosis, Huntington's disease, and multiple sclerosis [46]. It was reported that  
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24 some antioxidants, (Vit C and BHA) mediated the induction of NRF2 that is involved in the  
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26 regulation of OGG1 [46]. The human OGG1 promoter contains a putative NRF2 binding site and  
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28 NRF2 leads to OGG1 transcriptional activation [47,48]. In this study, we found that pesticide  
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30 treatment and in a more extent, polyphenols of honey extracts induced nuclear translocation of  
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32 NFR2 and a significant increase of OGG1 protein expression into the nucleus (*cf* Fig.6). Although  
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34 either oxidants or antioxidants induced NFR2-mediated nuclear OGG1 expression, increased DNA  
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36 repair activity was found only in cells treated with honey extracts. These findings suggest a  
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38 regulatory mechanisms at the post-transcriptional as previously described [49]. We can postulate  
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40 that pesticides may affect DNA repair enzyme activity by altering the redox-sensitive site(s) of  
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42 proteins, which may be prevented by polyphenols of honey extract incubation.  
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49 The effect of honey on pesticide-induced genotoxicity was than confirmed in vivo. An  
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51 altered DNA damage response (DDR) was found in a residential population long-term exposed to  
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53 low-level pesticides. In these subjects a reduced DNA repair activity was observed, respect to  
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55 subjects living in a pesticide-free area. Notably, the low DNA repair activity was further reduced in  
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57 the period of exposure to pesticides, which was associated with a dose-dependent increase of DNA  
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3 lesions (FPG-sites, ENDOIII-sites). The two-week honey supplementation significantly reversed  
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5 DNA repair activity and inhibited pesticide-induced DNA damage (*cf* Fig.7). There is an increasing  
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7 concern about chronic low-level pesticide exposure; a meta-analysis indicated that children exposed  
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9 to indoor insecticides would have a higher risk of childhood hematopoietic cancers [50]. Prolonged,  
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11 low-level pesticide exposure can also cause peripheral neurotoxicity, especially in sensory nerves  
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13 [51]. Thus, highlighting the importance of a diet-rich in polyphenols in the prevention of the  
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15 deleterious effect of chronic exposure to pesticides.  
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20 Taken together, pesticides, even at low doses, induce DNA damage through mitochondria  
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22 destabilization. DNA lesion accumulation occurs as consequence of a reduced DNA repair activity  
23  
24 induced by pesticide exposure. The honey containing polyphenols affects *in vitro* and *in vivo* the  
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26 DNA damage response, thus limiting the toxicity induced by pesticides.  
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## Conflict of interest

The authors declare no conflict of interest

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19 **Author contribution:** RA, MT, NM study design and subject enrolment; NM, SG, VC, FM,  
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21 performed experiments, data collection and analysis; MFC, performed the polyphenol profile  
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23 evaluation on honey extracts; BB, MB, MA, LS, blood and air sample collection, environmental  
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25 analysis, and contribute for materials and reagents.  
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## Legend to figures

**Figure 1-** Polyphenol profile of honey extracts, and their antioxidant capacity. **A)** Extracts from honey (acacia, Ext-1; chestnut, Ext-2; orange tree, Ext-3; woodland, Ext-4) were evaluated for their polyphenol types and content. The total polyphenol level for each extracts is shown in the insert. **B)** The antioxidant property of honey extracts was evaluated by Oxygen radical absorbance capacity (ORAC) assay, and the total antioxidant activity expressed as mM trolox (insert). The results are the mean  $\pm$  SD of three experiments.

**Figure 2-** Effects of pesticide exposure on cell proliferation, mitochondrial activity, and ROS formation. **A)** Human bronchial epithelial cells (BEAS-2B) and neuronal SHSY-5Y cell line (**B)** were treated with increased concentration (0-1000  $\mu$ M) of glyphosate (GLY) and Chlorpyrifos (CPF), and cell proliferation rate (left-panel), mitochondrial activity rate (right-panel) evaluated. Reactive oxygen species (ROS) were detected in BEAS-2B cells and SHSY-5Y cells after GLY and CPF exposure at 10  $\mu$ M and 100  $\mu$ M (**C**). The results are expressed as fold change respect to untreated cells (control). The results are the mean  $\pm$  SD of three experiments performed in duplicate. Comparisons among groups were determined by one-way ANOVA with Tukey post hoc analysis. The symbol ‘\*’ indicates significant differences with  $p < 0.05$ .

**Figure 3-** Effects of honey-extracts on pesticides-induced mitochondrial destabilization and ROS formation. BEAS-2B cells were 2 h pre-treated with honey extracts (5  $\mu$ g/ml) followed by 10  $\mu$ M and 100  $\mu$ M glyphosate (GLY, left-panel) or 10  $\mu$ M and 100  $\mu$ M chlorpyrifos (CPF, right-panel). Control cells received GLY or CPF treatment (10  $\mu$ M and 100  $\mu$ M). After 24 h of incubation, mitochondrial activity (**A**) and ROS formation (**B**) were evaluated. The results are the mean  $\pm$  SD of three experiments performed in duplicate. Comparisons among groups were determined by one-

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3 way ANOVA with Tukey post hoc analysis. The symbol “\*” indicates significant differences  
4 compared with untreated cells (GLY-0, CPF-0), the symbol “°” significance compared with  
5 control (Ctrl), with  $p < 0.05$ .  
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13 **Figure 4-** Effects of ROS inhibitors Tiron and NAC, and honey-extract to pesticide-induced ROS  
14 formation. **A)** BEAS-2B cells were treated with 10-100  $\mu\text{M}$  glyphosate (GLY) or 10-100  $\mu\text{M}$   
15 chlorpyrifos (CPF) in the presence or absence of Tiron (10 mM), NAC (10 mM), and honey extract  
16 (5  $\mu\text{g}/\text{ml}$ ). **B)** BEAS-2B cells were treated with 10-100  $\mu\text{M}$  GLY (left-panel) or 10-100  $\mu\text{M}$  CPF  
17 (right-panel) in the presence or absence of honey-extracts (5  $\mu\text{g}/\text{ml}$ ) and DNA damage evaluated as  
18 single strand breaks (SSBs), FPG and ENDOIII lesions. **C)** SHSY-5Y cells were treated with GLY  
19 (100  $\mu\text{M}$ ) or CPF (100  $\mu\text{M}$ ) in presence or absence of honey-extract (5  $\mu\text{g}/\text{ml}$ ) and DNA damage  
20 evaluated. The results are expressed as fold change of control of three independent experiments.  
21 Comparisons among groups were determined by one-way ANOVA with Tukey post hoc analysis.  
22 The symbol “\*” indicates significant differences compared with untreated cells (Ctrl), the symbol  
23 “°” significance between pesticide-treated cells and cell incubated with ROS inhibitors (Tiron,  
24 NAC) or honey extract, with  $p < 0.05$   
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44 **Figure 5-** Effect of honey-extracts on DNA repair activity. Human bronchial epithelial cells  
45 (BEAS-2B) and neuronal SHSY-5Y cell line were overnight incubated with honey extracts and the  
46 DNA repair activity was evaluated in cellular extracts (**A**) or as kinetics of DNA strand break  
47 rejoining in cells after exposure to hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 100  $\mu\text{M}$ ) (**B**). The kinetics of DNA  
48 repair was calculated as a percentage of residue damage respect to the time of maximum damage  
49 ( $T_n$ ). Residual damage (%) =  $100 \times [\text{DNA damage at time T after treatment } (T_n) - \text{DNA damage of}$   
50 untreated cells ( $T_0$ ) / DNA damage immediately after treatment ( $T_b$ ) - DNA damage of untreated  
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3 cells (T0)]. BEAS-2B and SHSY-5Y cells were treated with 10-100  $\mu$ M glyphosate (GLY) or 10-  
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5 100  $\mu$ M chlorpyrifos (CPF) for 24 h (C) or chronically exposed to both pesticides (D) in the  
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7 presence or absence of honey extract (5  $\mu$ g/ml, 24h), and DNA repair activity evaluated. The  
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9 symbol ‘\*’ indicates significant differences compared with control (Ctrl) and treated cells, with  $p <$   
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18 **Figure 6-** Cellular NFR2 activation and OGG1 expression are induced in response to pesticides and  
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20 honey extract treatment. Human bronchial epithelial cells (BEAS-2B) and neuronal SHSY-5Y cell  
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22 line were treated with glyphosate (GLY, 100  $\mu$ M) and chlorpyrifos (CPF, 100  $\mu$ M) with or without  
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24 honey extract (Ext-4) pre-incubation (2 h). The NFR2 and OGG1 nuclear translocation were  
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26 evaluated in BEAS-2B cells (A) and SHSY-5Y cells (B) by western blot (left-panel), and the levels  
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28 of the bands quantified by densitometry analysis (right-panel). Comparisons among groups were  
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30 determined by one-way ANOVA with Tukey post hoc analysis. The symbol ‘\*’ indicates significant  
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32 differences compared with untreated cells (Ctrl).  
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40 **Figure 7-** Effect of honey supplementation on the DNA damage response (DDR) in residents  
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42 chronically exposed to pesticides. A population chronically exposed to pesticides was enrolled at  
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44 three different periods of pesticide exposure: no pesticides-exposure, (P0, February-March), low-  
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46 exposure, (P1, October-November), and high exposure, (P2, May-June). In the P1 and P2 periods,  
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48 DNA repair activity (A), time to repair 50% of H<sub>2</sub>O<sub>2</sub>-induced DNA damage ( $t_{1/2}$ ) (B), FPG-sites (C)  
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50 and ENDOIII-sites (D) were evaluated in pesticide-exposed residents before (S0) and after two  
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52 weeks honey supplementation (50 g/day) (S1). Pesticide non-exposed subjects were used as  
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54 controls (CTRL). Comparisons among groups were evaluated by ANOVA repeated measure with  
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56 Sidak post-hoc test, with  $p < 0.05$ .  
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**Legend to supplementary figures**

**Supplementary Figure S1-** HPLC–UV chromatograms at 280 nm (**A**) and 330 nm (**B**) of the identified phenolic compounds and abscisic acids in buckwheat honey extract. Peaks: HMF: 5-hydroxymethylfurfural; (1) protocatechuic acid; (2) p-hydroxybenzoic acid; (3) p-ydroxyfenilacetic acid; (4) caffeic acid; (5) unidentified compound; (6) syringic acid; (7) p-coumaric acid; (8) ferulic acid; (9) isoferulic acid; (10) benzoic acid; (11) trans–trans abscisic acid; (12) cis–trans abscisic acid; (13) quercetin; (14) apigenin; (15) pinobanksin; (16) kaempferol; (17) isorhamnetin; (18) chrysin; (19) pinocembrin; (20) galangin.

**Supplementary Figure S2-** Effect of honey extract on over time pesticide-induced mitochondrial destabilization and ROS formation. BEAS-2B cells were treated with 10-100  $\mu$ M glyphosate (GLY, left-panel) or 10-100  $\mu$ M chlorpyrifos (CPF, right-panel) in presence or absence of honey extract (2 h pre-incubation), and mitochondrial activity (**A**) and ROS formation over time (**B**) evaluated. The results are expressed as fold change respect to untreated cells (control). The results are the mean  $\pm$  SD of three experiments performed in duplicate.

**Supplementary Figure S3-** Effects of honey-extracts on pesticides-induced mitochondrial destabilization and ROS formation in SHSY-5Y cells. SHSY-5Y cells were treated with 10-100  $\mu$ M glyphosate (GLY, left-panel) or 10-100  $\mu$ M chlorpyrifos (CPF, right-panel) in presence or absence of honey extracts (5  $\mu$ g/ml) and mitochondrial activity (**A**) and ROS formation (**B**) evaluated. The results are the mean  $\pm$  SD of three experiments performed in duplicate. Comparisons among groups were determined by one-way ANOVA with Tukey post hoc analysis. The symbol ‘\*’ indicates significant differences compared with untreated cells (CLY-0, CPF-0), the symbol ‘‘’’ significance compared with control (Ctrl), with  $p < 0.05$ .

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3 **Supplementary Figure S4.** Life-style and dietary habits of enrolled subjects. According to the  
4 questionnaire on life-style and dietary consumption, the dietary habits (**A**), frequency of food intake  
5 (**B**), and drinking (**C**) were evaluated.  
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13 **Supplementary Figure S5-** Pesticides evaluated in the air samples. According to the calendar of  
14 treatments a list of pesticide compounds was performed and their concentration detected in air  
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**Table 1-** Demographic and anthropometric characteristics of the recruited subjects, and biological/ environmental exposure to pesticides

A

Demographic/anthropometric indices	Pesticides exposed population (n=34)			Pesticides non-exposed population (n=40)		
	median	25°-75°	%	median	25°-75°	%
Age (years)	46	15-55		35	30-55	
Gender (Male/Female)			41/59			48/52
Weight (kg)	58	50-70		56	55-72	
Height (cm)	166	151-174		159	162-175	
BMI (Kg/m <sup>2</sup> )	22	20-23		21	20-22	
Smoking (yes/no)			22/78			12/82

B

Biological pesticide level	Periods of exposure to pesticides		
	P0	P1	P2
	Median (25°-75°)	Median (25°-75°)	Median (25°-75°)
TCP (µg/g creat)	2.1 (1.9-3.5)	3.2 (2.7-3.6)*	4.4 (3.3-5.0)*
Environmental pesticides level (ng/m <sup>3</sup> )			
Chlorpyrifos ethyl	-	-	5.1 (0.7-7.4)
Difenoconazol	-	-	2.2 (0.7-2.8)
Fluazinam	-	-	18.3 (17.6-19.4)
methoxyfenozide	-	-	0.5 (0.3-0.8)

TCP= 3,5,6-trichlor-2-piridinol; creat=creatinine

P0, no-exposure period; P1, low-exposure period; P2, high-exposure period

Data are shown as median [25°percentile-75° percentile] for continuous variables and percentages for categorical variables. \*P1 and P2 versus P0, p<0.05

Figure 1

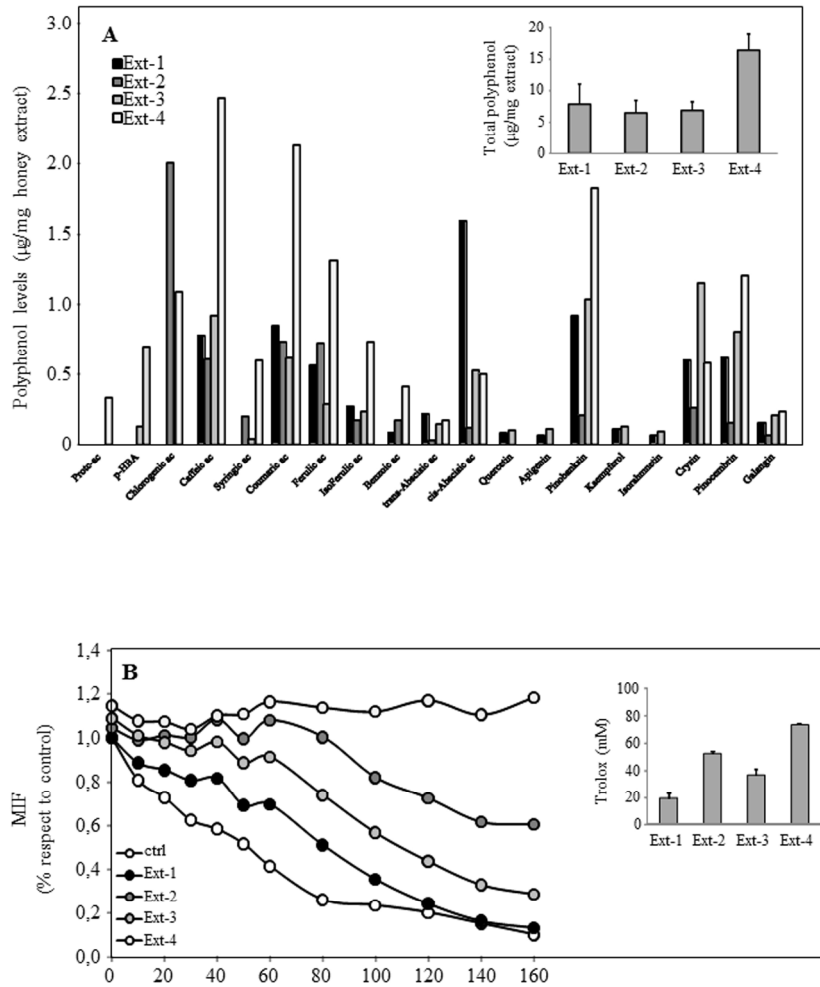


Figure 1  
254x338mm (300 x 300 DPI)

Figure 2

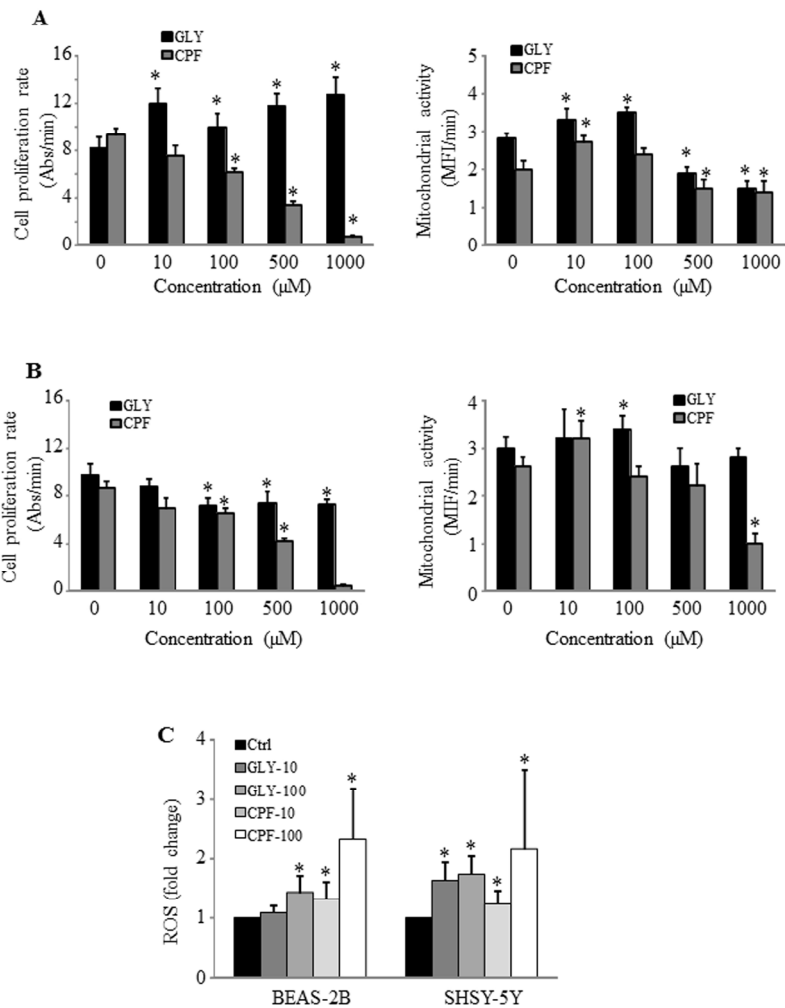


Figure 2  
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Figure 3

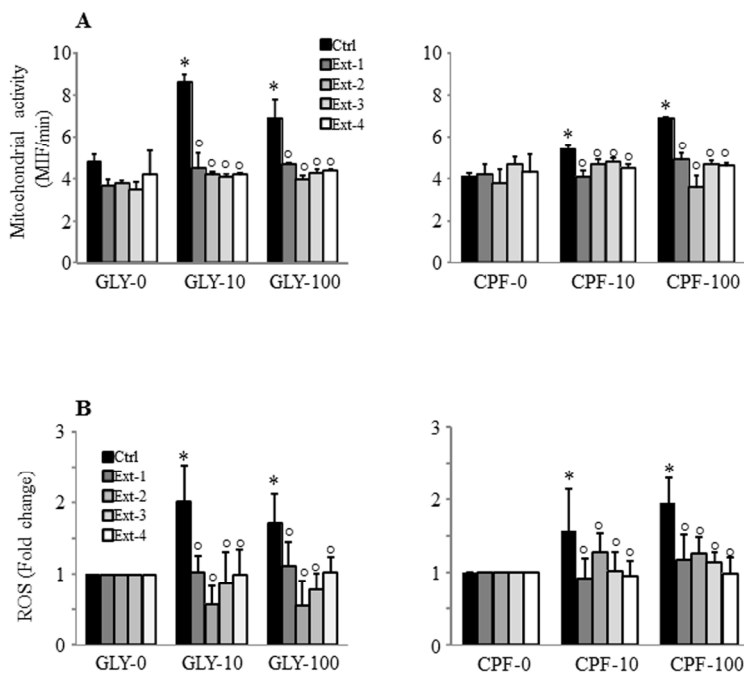


Figure 3  
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Figure 4

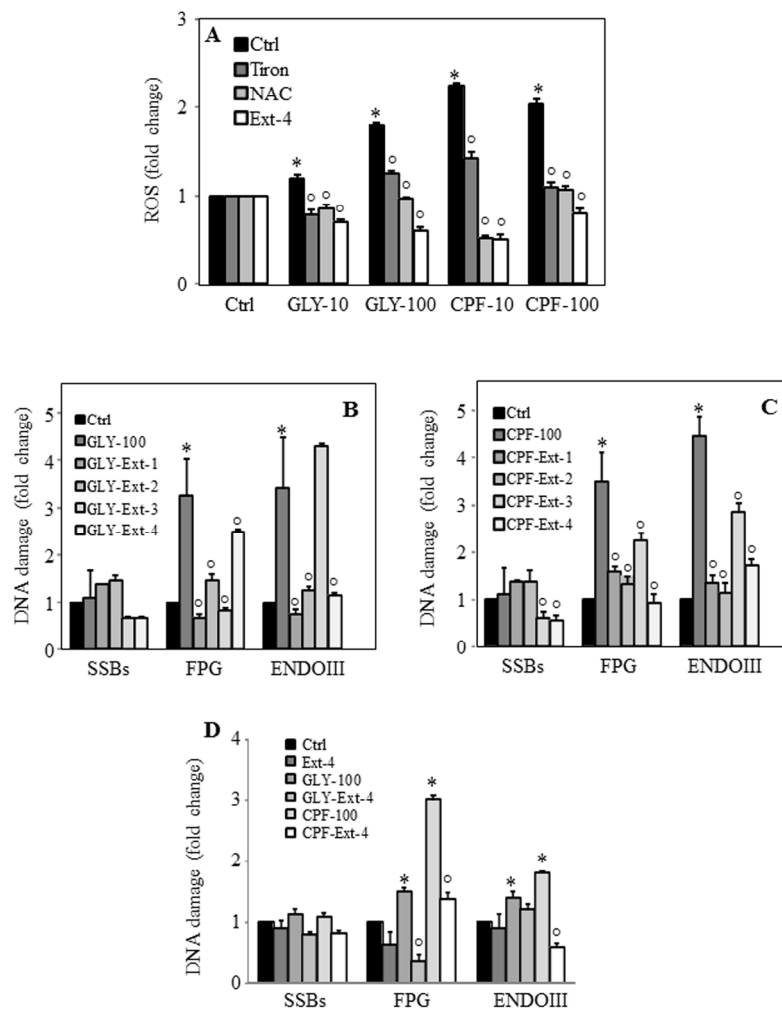


Figure 4  
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Figure 5

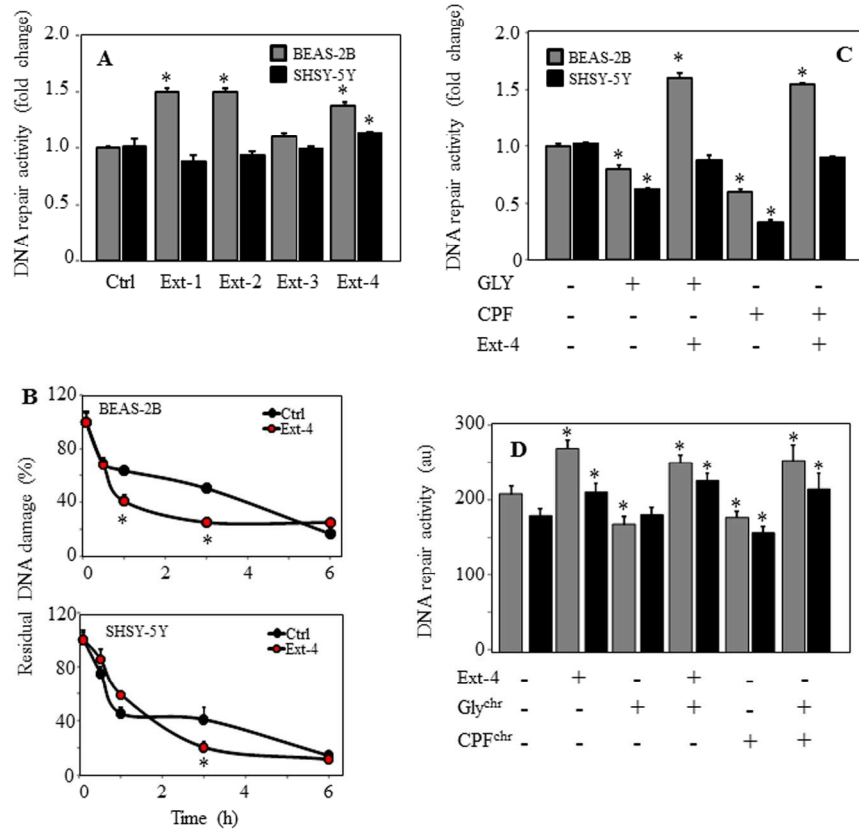


Figure 5  
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Figure 6

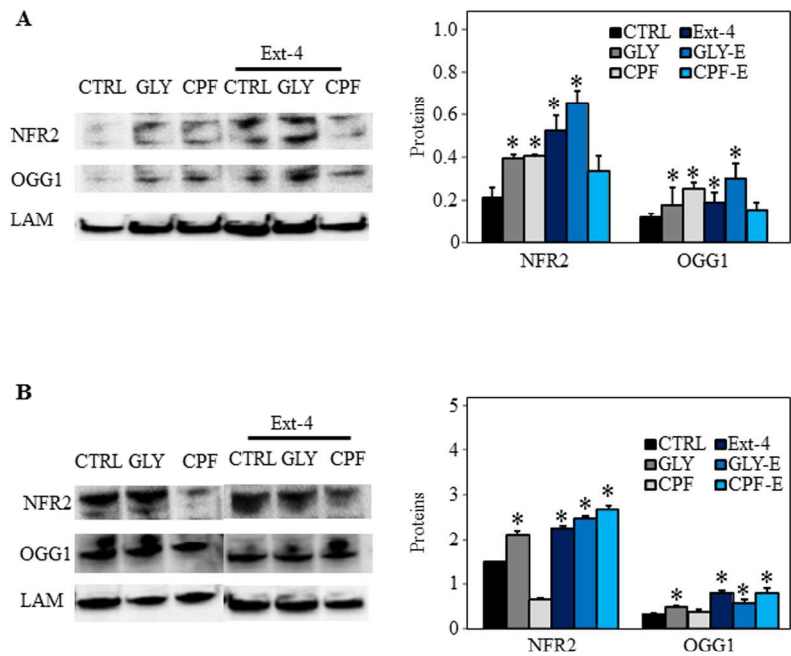


Figure 6  
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Figure 7

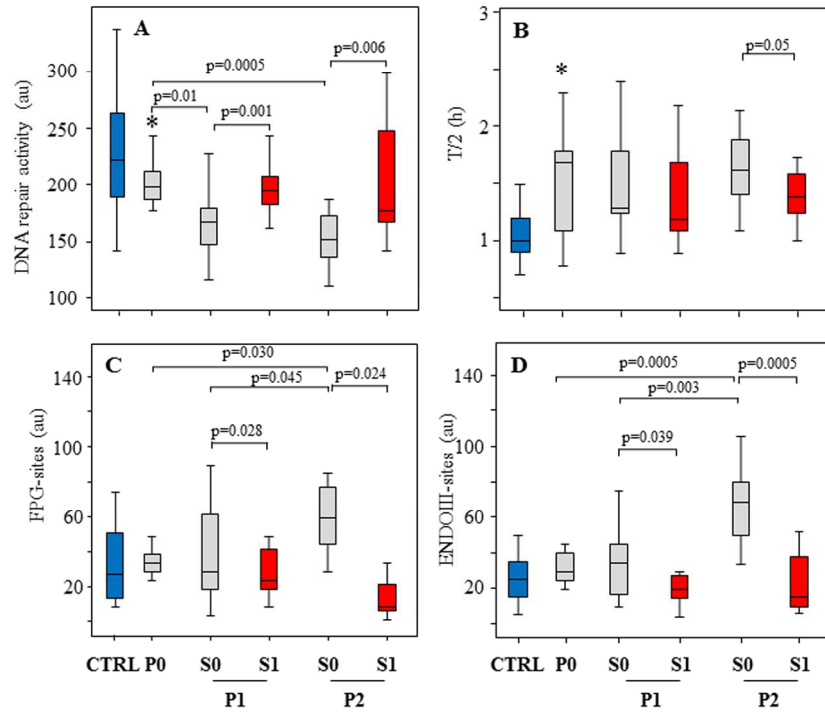
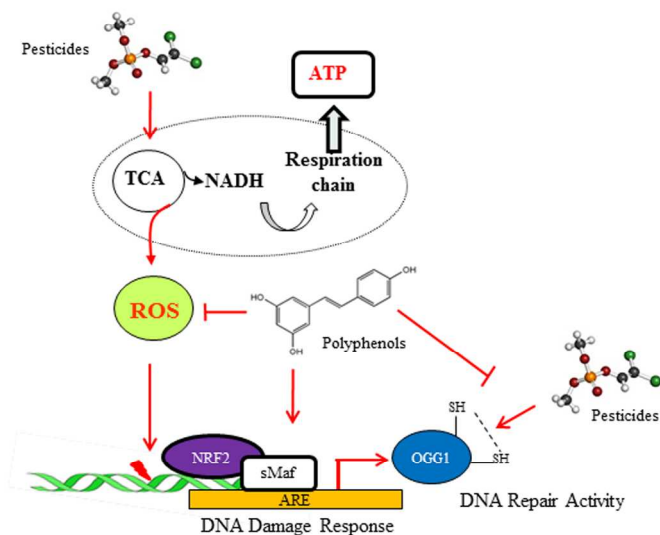


Figure 7  
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Graphical abstract



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