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Genomic instability induced by α-pinene in Chinese hamster cell line

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Here, we report the effects of exposure of mammalian cells to α-pinene, a bicyclic monoterpene used in insecticides, solvents and perfumes. Morphological analysis, performed in V79-Cl3 cells exposed for 1 h to increasing concentrations (25 up to 50 μM) of α-pinene, indicated a statistically significant increase in micronucleated and multinucleated cell frequencies; apoptotic cells were seen at 40 and 50 μM. This monoterpene caused genomic instability by interfering with mitotic process; in fact, 50% of cells (versus 19% of control cells) showed irregular mitosis with multipolar or incorrectly localised spindles. Cytogenetic analysis demonstrated high-frequency hypodiploid metaphases as well as endoreduplicated cells and chromosome breaks. Clastogenic damage was prevalent over aneuploidogenic damage as demonstred by the higher proportion of kinetochore-negative micronuclei. Alkaline comet confirmed that monoterpene exposure caused DNA lesions in a concentration-dependent manner. This damage probably arose by increased reactive oxygen species (ROS) production. In order to assess the generation of ROS, the cells were incubated with CM-H_2DCFDA and then analysed by flow cytometry. Results demonstrated an increase in fluorescence intensity after α-pinene treatment indicating increased oxidative stress. On the whole, these findings strongly suggest that α-pinene is able to compromise genome stability preferentially through mitotic alterations and to damage DNA through ROS production.

Introduction

Essential oils are natural mixtures that have numerous allelochemical functions between plants and between plants and predators, such as attraction or repulsion. They have been used since ancient times as natural remedies (1); today, they are present in household detergents, cleaning products, soaps, perfumes and cosmetics; they are also added in fruit juices, soft drinks and ice creams as flavouring agents. Some essential oils and their constituents have antibacterial and antiviral properties, as well as antitumour activity as pro-oxidant agents (2,3). Although some of the constituents have been found to exert antimutagenic activity, such as α-bisabolol, a sesquiterpene alcohol found in the chamomile oil (4), others gave positive results in the Ames test, such as mentone of the peppermint essential oil and terpineol of the cajuput and pine oils (5,6); some were found to induce chromosomal aberrations and endoreduplications in V79 cells, such as eugenol of clove and marjoram oils (7).

Among the different components of essential oils, the monoterpenes are the most common molecules (∼90%) and they are generally considered as safe substances. However, some monoterpene, such as pulegone, have been assessed to be hepatocarcinogenic in rats and mice (8) and d-limonene is still under study as the genotoxicity data so far have been inconclusive and contradictory (9).

α-Pinene, a bicyclic and hydrophobic monoterpene, is widely diffused in the plant kingdom; in fact, it is present not only in essential oils of pine trees (Pinus) but also in citrus fruits and in other aromatic plants, flowers and herbs (3,10,11). Although α-pinene is commonly used in insecticides, solvents, perfumes and is a food additive, information on the actual levels of this component in the different commercial products is still scarce (12), even though the Joint FAO/WHO Expert Committee on Food Additives had requested since 2005 that the industry provides precise data on the use levels of α-pinene and other flavouring agents that may be used in food products (13).

As research in the field of the safety evaluation of α-pinene is limited (4,13,14) despite its widespread use, the present investigation was designed to investigate the cytotoxic effects induced by acute exposure to α-pinene of V79-Cl3 Chinese hamster cells. Moreover, after the morphological analysis of nuclear abnormalities, mutagenesis tests such as micronucleus and comet assays were utilised to assess its potential genotoxicity.

Materials and methods

Cell culture and chemicals

The V79-Cl3 cell line, subcloned in our laboratory from V79 Chinese hamster cell line from which does not differ for plating efficiency, spontaneous mutation rate or modal chromosome number, has a doubling time of 12–15 h. Cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Paisley, UK), supplemented with 5% foetal calf serum (Invitrogen), penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained at 37°C in a 5% CO_2 humidified incubator; they were checked routinely for stability in the modal chromosome number; the absence of mycoplasma contamination was checked by Mycoplasma detection Kit (Roche Applied Science, Mannheim, Germany).

α-Pinene (CAS No. 80-56-8; purity 98%) was purchased from Sigma–Aldrich (St Louis, MO, USA). Working solutions were prepared immediately before use by dissolving α-pinene in complete medium supplemented with 0.5% dimethyl sulfoxide (DMSO) (Sigma–Aldrich); this amount of DMSO, through the use of appropriate controls, was shown to have no significant effect on cell growth and morphology.

Trypan blue exclusion and clonogenic assays

Cells (3×10^5 per dish) in exponential growth were exposed to arbitrarily chosen concentrations (0, 25, 30, 35, 40 or 50 μM) of α-pinene for 1 h. At the end of treatments, the cells were washed twice with Hank's salt solution,

collected by trypsinization and centrifuged for 5 min at 1000× *g*. Treated and untreated cells, suspended in serum-free medium, were stained for 5 min with 0.4% Trypan blue (Sigma–Aldrich) (v/v) and counted using a Burker chamber under a light microscope with an ×20 magnification. The Trypan blue dye is normally taken up by non-viable cells but not by viable cells because it only penetrates through damaged cellular membranes. Cell viability was expressed as the percentage of viable cells compared to the total number of counted cells. Then, for each sample, a number of cells sufficient to provide 300 viable cells per dish were plated in triplicate in fresh medium and incubated for 10 days; colonies were stained with 0.1% methylene blue. Clonal-forming ability was expressed as percentage of untreated cells.

Three independent experiments were carried out and data are reported as means ± SD.

Apoptosis assay

Exponentially growing cells (2×10^4) on 18 × 18 mm glass coverslips, placed in 60 mm petri dishes, were treated with 0, 35, 40 or 50 μM α-pinene for 1 h; after two washes with Hank's salt solution, they were prefixed with methanol added to the culture medium in a 1:1 ratio for 5 min at room temperature and then fixed with methanol for 7 min, both immediately after treatment and after post-treatment incubation in fresh medium for 3 h. Cells were assayed for apoptotic chromatin condensation by the TdT-Frag-El kit (Trevigen, Gaithersburg, MD, USA), according to the recommendations of the manufacturer. By this methodology, apoptotic cells resulted coloured brown and normal cells blue-green. At least 200 cells were blindly scored for each sample.

Morphological observations/micronucleus test

Cells (2×10^4) were plated on 18 × 18 mm glass coverslips and placed in 60 mm petri dishes in order to evaluate the presence of micronucleated and multinucleated cells, according to a previous reported protocol (15). After 18 h, the cells, treated for 1 h with increasing concentrations (0, 25, 30 or 35 μM) of α-pinene in complete medium, were washed, fixed as reported above and stained with 2.5% Giemsa (Gurr R66, BDH, Poole, UK), both immediately and after post-treatment incubation in fresh medium for 3 and 6 h. At least 1000 cells were examined each time at each concentration and three independent experiments were performed to determine abnormal cell frequencies. The slides were coded and scored blind under a Zeiss Axiophot light microscope at ×1350 magnification under oil immersion.

For classification of micronuclei as kinetochore-positive (k^+) or -negative (k^-), some coverslips with cells exposed to 0 or 35 μM α-pinene for 1 h were submitted to indirect immunostaining technique both immediately and after post-treatment incubation in fresh medium for 3 and 6 h. After three washes of 5 min in phosphate buffered saline (PBS, Invitrogen) and two washes of 2 min in PBS + 1% bovine serum albumine (BSA, Sigma–Aldrich), the cells were incubated in a humidified chamber over night at 4°C with antinuclear antibody (Antibodies Incorporated, Davis, CA, USA). At the end of the incubation, the cells were rinsed three to four times with PBS + 1% BSA and incubated with fluorescein (FITC)-conjugated anti-human IgG antibody (Sigma–Aldrich), diluted 1:30 in the same washing solution, for 1 h at 37°C. After washing, the preparations were mounted in antifade solution (1 mg/ml *p*-phenylenediamine dihydrochloride in one part PBS and nine parts 97% glycerol, pH 8.0) containing 2 μg/ml of propidium iodide (Sigma–Aldrich) for counterstaining. To evaluate the presence of micronuclei with or without kinetochore (k^+ or k^-), enough cells were scored to reach 100 micronuclei per sample by utilising a Nikon fluorescence photomicroscope equipped with an HBO 100 W mercury lamp and a suitable filter. Then, k^+ or k^- micronuclei relative proportion was calculated by multiplying the percentage of micronuclei found in each sample by proportion of micronuclei k^+ or k^-.

Chromosome aberration tests

Cytogenetic observations were performed in cells exposed to 0, 25, 30 or 35 μM α-pinene at the end of the 1-h treatment and processed according to the previous reported procedure (16). Two independent experiments were carried out for each concentration and 100 metaphases were scored for each sample to determine the number of chromosomes and chromosome breaks per cell. The mitotic indices were estimated by counting at least 1000 cells and the presence of endoreduplicated metaphases was evaluated by scoring 1000 metaphases. The slides were coded and scored blind.

Indirect immunostaining

For the microtubule analysis, cells exposed to 0 or 35 μM α-pinene for 1 h, washed and fixed as reported above, were observed immediately after treatment. After washing in PBS + 3% BSA with 0.1% Triton X-100 (BDH), the cells were incubated in a humidified chamber for 30 min at 37°C with mouse anti-ß-tubulin antibody (Sigma–Aldrich), diluted 1:50 in the same washing solution. After three rinses, the cells were incubated for 30 min at 37°C with fluorescein (FITC)-conjugated anti-mouse IgG (Sigma–Aldrich) diluted 1:32. After washing, the preparations were mounted in antifade solution. For each sample, at least 100 metaphases were observed under a confocal laser scanning photomicroscope (Olympus FV-300) equipped with argon (488 nm) and helium/neon (543 nm) lasers.

Alkaline comet assay

The comet assay was performed according to Singh *et al.* (17) with few modifications. Briefly, exponentially growing cells were treated with 0, 25, 30 or 35 μM α-pinene for 1 h; after trypsinization, cells were evaluated for viability by trypan blue exclusion, pelleted by centrifugation at 800 r.p.m. for 10 min, resuspended in PBS (2×10^5 cells/10 μl, final concentration) and mixed with 65 μl 0.7% low melting agarose (LMA; Bio-Rad, Segrate, Italy). They were then layered onto slides (GelBond film; Sigma), previously coated with a layer of 0.5% normal melting agarose (Fisher Molecular Biology, Trevose, PA, USA) and covered with another layer of LMA. The agarose suspension was covered with a coverslip and placed at 4°C for 10 min. The coverslips were gently removed and the slides were submersed into lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10.0) for 1 h in the dark at 4°C. After lysis, the slides were placed for 15 min in a horizontal electrophoresis gel tray containing fresh alkaline buffer (300 mM NaOH, 1 mM Na_2EDTA, pH > 8) and then subjected to an electric field of 0.7 V/cm in the dark at 4°C for 20 min. Following electrophoresis, the microgels were neutralised in 0.4 M Tris–HCl (pH 7.5), dehydrated in methanol for 2 min and allowed to dry at room temperature. The DNA was stained with ethidium bromide (2 μg/ml) (BDH) and visually examined by a Nikon fluorescence microscope, equipped with an HBO 100 W mercury lamp and a suitable filter. DNA damage was quantified by tail length (TL) and tail moment (TM) values calculated by CASP (Comet Assay Software Project). The TL is the distance from the middle of nucleoid core to the end of the tail; TM is defined as the product of the percentage of DNA in the tail distribution and TL (18).

Data were derived from three independent experiments, with at least 50 cells per experiment (from two replicate slides each).

Reactive oxygen species evaluation

Intracellular reactive oxygen species (ROS) levels were evaluated by flow cytometry using 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Molecular Probes Inc., Eugene, OR, USA) as a probe. The CM-H_2DCFDA penetrates into cells and is hydrolysed by cellular esterase to 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, which is further oxidised by intracellular ROS into a strong fluorescent compound, dichlorofluorescein, that can be detected by Fluorescence Activated Cell Sorting (FACS). After treatment with 0, 25, 30, 35, 40 and 50 μM α-pinene for 1 h, the cells were washed twice with Hank's Balanced Salt Solution (HBSS) and then incubated with 25 μM of CM-H2DCFDA for 1 h. After washing in HBSS, the cells were harvested by trypsinisation, resuspended at 1×10^6 cells/ml in HBSS and immediately subjected to FACScan flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA) with excitation and emission settings of 488 and 530 nm, respectively. Three independent experiments were performed and the quantification of ROS levels for each sample was determined by dividing the mean fluorescence value for the treated sample with the mean fluorescence value for the untreated sample.

Statistical analysis

Nuclear and chromosome abnormality frequencies, ROS levels and the relative proportions of k^+ or k^- micronuclei in treated cells were compared with those observed in untreated cells using the Student's *t* test; $P < 0.05$ was considered significant.

Tail DNA parameter frequencies, which do not follow a Gaussian distribution (19), were processed with the Kruskal–Wallis non-parametric test to display a possible dose–effect relationship.

Results

Survival analyses

Cell survival after 1 h of treatment as measured by the trypan blue exclusion test and colony-forming ability showed concentration-related responses over the concentration range tested (25–50 μM) of α-pinene. In particular, the trypan blue exclusion test indicated that cell survival was 49.33 ± 1.15% at 40 μM and 39 ± 1.73% at 50 μM of monoterpene (Figure 1A). The clonogenic assay, performed on viable cells, showed

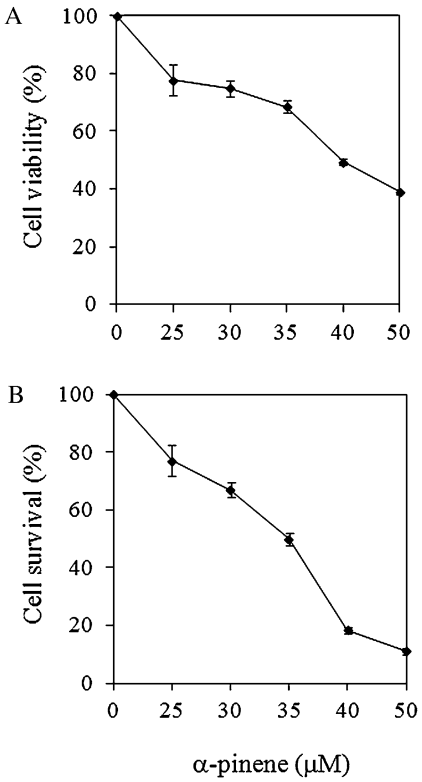

Fig. 1. Cell viability by trypan blue exclusion test (**A**) and cell survival by clonogenic assay (**B**) in increasing concentrations of α-pinene. Frequencies are expressed as the percentage of untreated cells. Data are presented as the mean ± SD percentages of three independent experiments.

a stronger effect at concentration of ≥30 μM, with the colony formation capability being 11.02 ± 1% at the highest dose of 50 μM (Figure 1B).

Apoptosis evaluation

The apoptotic assay demonstrated that, in comparison with the untreated cells, TdT-positive cells frequency was significantly different ($P < 0.05$) immediately after treatment with 40 or 50 μM of α-pinene and became very significant ($P < 0.005$) after 3 h of recovery post-treatment (Figure 2). Thus, the concentrations of 40 and 50 μM were excluded from those experiments directed towards the evaluation of the genome damage.

Morphological observations/micronucleus test

The results of morphological investigations performed up to 6 h after release from treatment with 0–35 μM of α-pinene are shown in Figure 3.

Multinucleated cell frequencies, estimated at the end of treatments for each concentration, significantly increased in comparison with the untreated cells ($P < 0.001$). After 3 h of recovery, these frequencies increased in values ($P < 0.0001$) and, after 6 h of recovery, decreased, although they were still statistically significant (Figure 3A).

Micronucleated cell frequency immediately after exposure was significantly greater than in untreated cells for the 30 μM concentration of α-pinene ($P < 0.005$) and it was highly statistically significantly for the 35 μM concentration ($P < 0.0001$). After 3 h of release from treatment, the frequency of micronucleated cells was evidently concentration dependent; in fact, the frequency induced by 25 μM was statistically

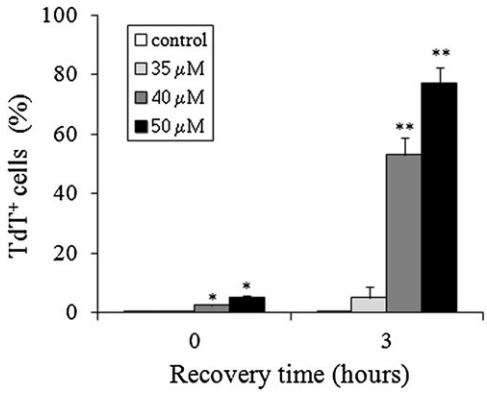

Fig. 2. Frequency of TdT-positive cells immediately after treatment with selected α-pinene concentrations and after 3-h recovery. *$P < 0.05$ versus control; **$P < 0.005$ versus control, according to the Student's t test.

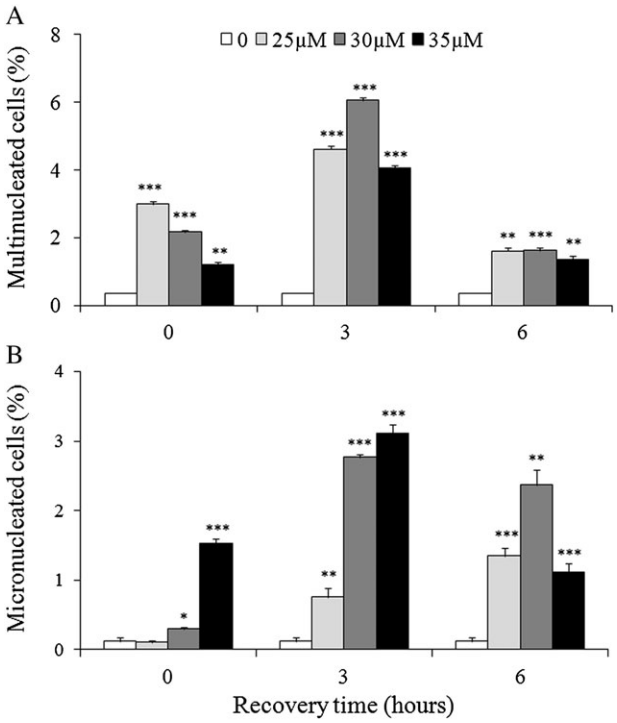

Fig. 3. Frequencies of multinucleated (**A**) and micronucleated (**B**) cells after 1-h treatment with increasing concentrations of α-pinene and during recovery from treatments. Bars represent standard deviation of three independent experiments. *$P < 0.005$ versus control; **$P < 0.001$ versus control; ***$P < 0.0001$ versus control, according to the Student's t test.

significantly ($P < 0.001$) and that induced by 30 or 35 μM was highly statistically significantly ($P < 0.0001$) increased. After 6 h of release from treatment, these frequencies decreased but were still very statistically significant ($P < 0.001$) (Figure 3B).

For classification of micronuclei as kinetochore-positive (k^+) or -negative (k^-), cells exposed to 0 or 35 μM α-pinene for 1 h were examined using the indirect immunostaining technique both immediately and after post-treatment incubation in fresh medium for 3 and 6 h. In comparison with untreated cells, k^- micronuclei relative proportion showed a 17-fold increase (versus 9-fold for k^+ micronuclei) immediately after the exposure and a 30-fold increase (versus 25-fold for

k^+ micronuclei) after 3-h recovery; after 6-h recovery, the k^- micronuclei relative proportion became ~9-fold higher (versus 14-fold for k^+ micronuclei) (Figure 4).

Chromosome aberration tests

Results obtained from cytogenetic analysis performed immediately after treatment with α-pinene revealed that the frequency of cells with modal chromosome number decreased from 60% (control) to <20% at 30 and 35 μM. The frequency of cells with chromosome breaks as well as the frequency of endoreduplicated cells increased in a concentration-related manner (Table I).

Immunostaining

The indirect immunostaining technique with anti-β-tubulin antibody was performed on cells treated with 0 or 35 μM α-pinene. Observations carried out under a confocal laser microscope showed irregular mitoses with multipolar or incorrectly localised spindles (50 versus 19% in the untreated cells), immediately after the 1-h exposure. Figure 5 shows the focal plane sequences of an untreated cell (A) and of an α-pinene-treated cell (B).

Comet assay

In order to quantify directly the DNA damage in individual cells, the alkaline comet or single-cell gel electrophoresis (SCGE) was performed. Quantitative analysis, performed by the CASP software, indicated that monoterpene induced DNA lesions. In comparison to the control, TL and TM increased significantly with increasing doses of α-pinene in a concentration-dependent manner (Figure 6). Distribution of DNA damage, measured as TM, showed that 86.9% of untreated cells had a TM ranging from 0 to 1.5 μm, whereas 12, 96 and 100% of cells treated with 25, 30 or 35 μM of α-pinene, respectively, showed a TM >20 μm, an evident sign of severe DNA damage (Figure 7).

ROS levels

Intracellular ROS levels were detected in cells treated with α-pinene (0–50 μM) by measuring through flow cytometry their ability to oxidise the fluorogenic dye CM-H_2DCFDA. Compared with the FACS histogram for untreated cells, histograms for cells examined after each treatment showed a rightward shift, indicating a more extensive oxidation of the dye increasing with α-pinene concentrations. The quantification data of ROS levels were always significantly different from the control value ($P < 0.0001$) (Figure 8).

Discussion

Humans are naturally used to live in an environment in which α-pinene is present as a normal constituent of atmosphere and a common ingredient in several foods. Several studies report that when oral and inhalation exposures are combined, the average total daily exposure in an urban or rural environment is in the range of 10–30 mg (20). On the basis of estimated current intake, the European Food Safety Authority (EFSA)

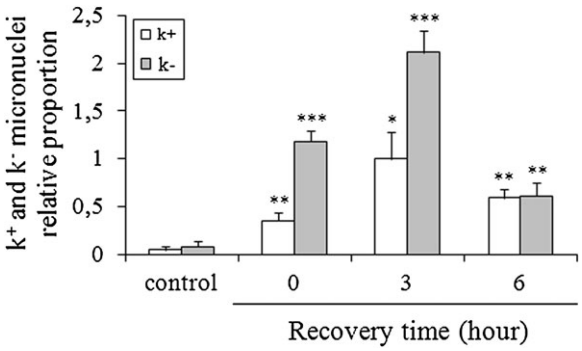

Fig. 4. Relative proportion of micronuclei with kinetochore (k^+) and without kinetochore (k^-) in untreated cells (control) and in cells examined after treatment with 35 μM α-pinene and after recovery. Bars represent standard deviation of three independent experiments. *$P < 0.05$ versus control; **$P < 0.01$ versus control; ***$P < 0.001$ versus control, according to the Student's *t* test.

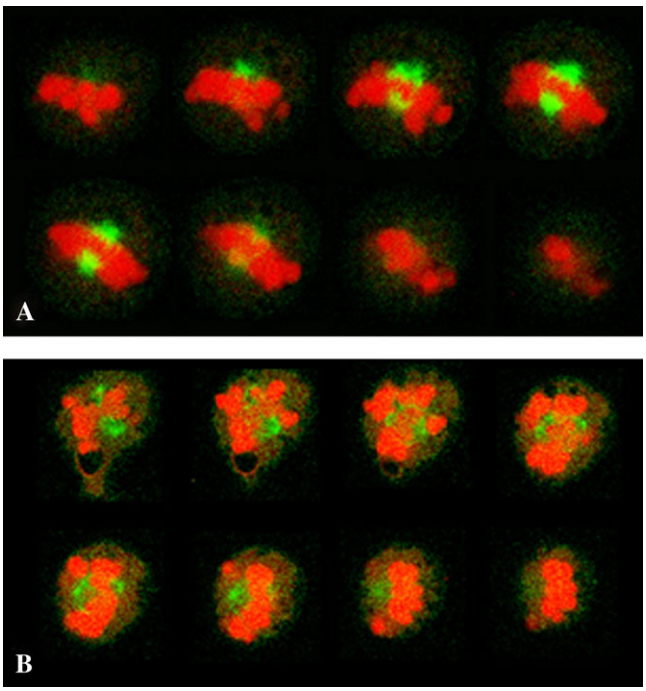

Fig. 5. Representative focal plane sequences of dividing cells under a confocal laser scanning photomicroscope at ×100 magnification. (**A**) untreated cell and (**B**) 35 μM α-pinene-treated cell with the chromosomal mass (red) wrongly set around the mitotic spindle that presents more than two poles (green).

Table I. Cytogenetic findings in untreated and α-pinene-treated V79-Cl3 cells

α-pinene (μM)	Mitotic index (%)	Cells with 21 chromosomes (%)	Cells with chromosome breaks (%)	Endoreduplicated cells (%)
0	49.5 ± 3.5	60.5 ± 0.7	0 ± 0	0 ± 0
25	34.0 ± 2.8	39.5 ± 2.1***	4.5 ± 0.7**	0.5 ± 0.7
30	33.0 ± 2.8	16.0 ± 1.4****	7.5 ± 0.7***	3.5 ± 0.7**
35	22.5 ± 0.7	17.0 ± 2.8****	11.0 ± 2.8*	4.0 ± 1.4*

*$P < 0.02$; **$P < 0.005$; ***$P < 0.001$; ****$P < 0.0001$, according to the Student's *t*-test.

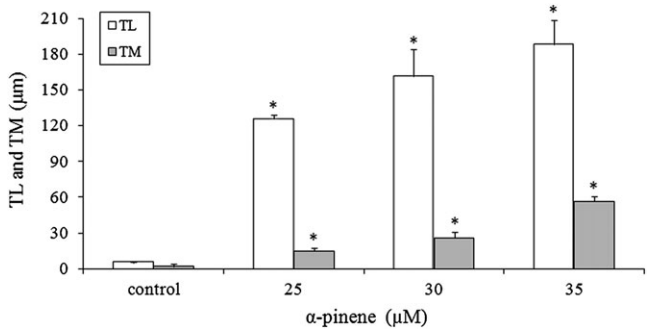

Fig. 6. Effects of selected α-pinene doses on the TL and TM by comet assay. Data represent the means ± SD from the experiments performed in triplicate. *$P < 0.001$, in agreement with the Kruskal–Wallis test.

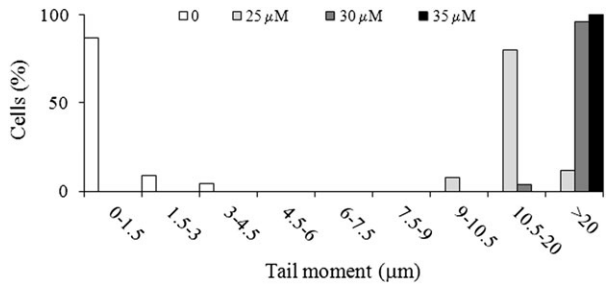

Fig. 7. Extent and distribution of DNA damage measured as TM of a representative experiment with selected α-pinene doses. Damaged cells were ordered according to increasing TM categories.

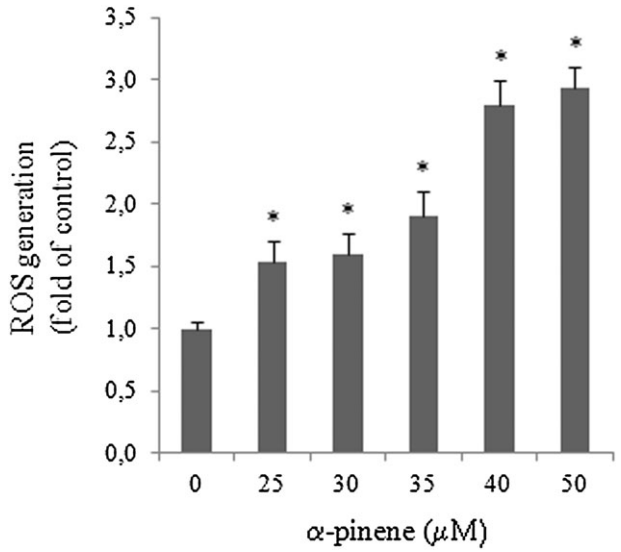

Fig. 8. Relative quantification of ROS levels after treatment with increasing concentrations of α-pinene. Data are normalised with respect to control and presented as the mean ± SD of three independent experiments. *$P < 0.0001$ versus control, according to the Student's *t* test.

concluded that α-pinene would not present a safety concern and established that the acceptable daily intake (ADI) is 'not specified', similarly to d-limonene which shares structural analogies with α-pinene (21). However, for occupationally exposed workers, the threshold limit value (TLV) is 20 p.p.m. averaged over an 8-h work shift (22) as it is known that α-pinene inhalation could cause palpitation, dizziness, nervous disturbances, chest pain, bronchitis or nephritis (23). Moreover, α-pinene is said to provoke irritation in case of eye contact or ingestion (24).

A number of studies demonstrated the genotoxicity of essential oils containing α-pinene by evaluating their capability of inducing chromosome aberrations and sister chromatid exchanges in human lymphocytes, and somatic mutations and recombination in *Drosophila melanogaster* (25), somatic crossing over in *Aspergillus nidulans* (26), DNA strand breaks in V79 cells (27) and micronuclei and chromosome aberrations in rodents (28). On the contrary, to our knowledge, only one paper has been published about the genotoxic effects caused by this monoterpene alone in cultured mammalian cells; in this case, α-pinene, placed in an emission chamber to generate a test atmosphere, did not lead any adverse effects in exposed cells (29).

In the present paper, we report the effects of exposing V79-Cl3 cells to acute treatments with α-pinene at concentrations that were below the daily intake established in 2005 for Europe and USA (13). A relevant finding of this study is that the biological effects are very often concentration-dependent.

In order to obtain information on α-pinene cytotoxicity, two different assays—the trypan blue exclusion test and the colony-forming ability—were consecutively used. This two-tier approach enabled us to know that viable cells lost their colony formation capability in concentration-related manner. In particular, cells still viable immediately after treatment with the higher concentrations (40 or 50 μM), were so severely damaged that they triggered an apoptotic process which lead to death in 48 or 72% of cells within 3 h, while cells treated with the lower concentrations (up to 35 μM), although damaged, continued to proliferate. The small difference between 35 and 40 μM seemed to constitute a boundary between cell life and death, the former concentration representing a threshold over which cells were not capable to sustain the damage probably because they had exhausted their defense. For these reasons, the higher concentrations were excluded from tests performed to evaluate the α-pinene capability of inducing genome damage. The concentration of 35 μM was utilised as a representative one for immunofluorescence tests performed to interprete the results from morphological and cytogenetic analyses.

On the whole, our results demonstrated that α-pinene is able to compromise genome stability both directly through mitotic spindle alterations that lead to disordered chromosome segregation and indirectly through ROS production that induces DNA damage.

The detection of multinucleated cells as well as of kinetochore-positive micronucleated cells after α-pinene exposure was in good agreement with the finding of irregular mitoses with multipolar or incorrectly localised spindles and with the presence of cells having a lower chromosome number than untreated cells. The strong interference with mitotic apparatus was confirmed by the detection of endoreduplicated cells, the presence of which is a very rare occurrence in untreated V79-Cl3 cells (0.39%) (16). Thus, α-pinene seems to act like colchicine or taxol both in affecting microtubule polymerisation and in producing endoreduplicated cells (30) and can be regarded as an aneugen.

Other remarks can be made by examining the finding of the concentration-dependent increase of the micronucleated cells frequency after 3 h of release. Taking into account that the V79-Cl3 cell line is an asyncronous cell population, morphological

observations concerned the part of cells which had gone beyond the M-phase; thus, a greater number of cells could be found after 3 h of release than at the end of the exposure. If this interpretation is correct, this signifies that α-pinene is able to penetrate into the cell during the exposure and its effects persist, in spite of washing, at least for the following 3 h. This hypothesis is supported by the increased frequency of kinetochore-positive micronuclei that arise from lagging chromosomes, as well as kinetochore-negative micronuclei that arise from acentric chromosomal fragments. Thus, α-pinene can be considered both an aneugen and a clastogen.

The clastogenic activity of α-pinene, documented by the presence of kinetochore-negative micronuclei and DNA strand breaks (seen by chromosome aberration and comet assays), was most likely related to α-pinene ability to cause oxidative damage by enhanced generation of ROS as it has been reported in root tissue (31). We found a concentration-dependent increase of ROS, which at higher concentrations (40 and 50 μM) led to apoptosis. A similar occurrence has been reported in murine melanoma cell line (32).

In the cellular model we used, α-pinene results in a chemical of concern as, at the lower doses (i.e. up to 35 μM), it makes the cells genomically unstable but does not affect considerably their survival. It will be of interest to know what is the fate of cells made unstable by α-pinene because the capability of proliferating after treatment implies the possibility of perpetuating the effects of past injury, as we previously demonstrated in cells exposed to arsenic (33). On the other hand, our results provide first information about the α-pinene capability of promoting an early apoptotic process at the higher concentrations used here (i.e. 40 or 50 μM) in a non-tumour cell line and constitute a contribution to the comprehension of the mode of biological action of α-pinene as they highlighted the effects of the *in vitro* exposure on genome stability and integrity. Therefore, it would be opportune to continue the studies in order to ascertain that α-pinene, commonly used as non-toxic compound, is really a safe substance.

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