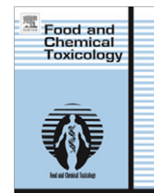
Food and Chemical Toxicology 50 (2012) 4051–4061

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox



Genetic toxicology evaluation of essential oil of *Alpinia zerumbet* and its chemoprotective effects against H_2O_2-induced DNA damage in cultured human leukocytes

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A R T I C L E I N F O

Article history:
Received 13 September 2011
Accepted 7 March 2012
Available online 17 March 2012

Keywords:
Alpinia zerumbet
Antimutagenesis
Chemoprevention
Antioxidative actions

A B S T R A C T

Essential oil (EO) of *Alpinia zerumbet* leaves, at non-toxic concentrations (50–300 μg/mL), did not induce genotoxicity in human leukocytes. However, at the highest concentration (500 μg/mL) tested caused a reduction in cell proliferation and viability, and an increase in DNA damage. Moreover, *in vivo* experiments showed that EO (400 mg/kg) did not exert mutagenicity on peripheral blood cells and bone marrow in mice. In DPPH test, EO showed scavenging effects against DPPH radicals, and other free radicals (determination of intracellular GSH and lipid peroxidation assays). Furthermore, EO was able to reduce the intracellular levels of ROS, and prevented leukocytes DNA against oxidative damage. The ability of EO to reduce H_2O_2 toxicity was observed only when cells were treated with EO during and after exposure to H_2O_2. With the co- and post-treatment procedures, EO decreased the frequency of apoptotic and micronucleated leukocytes as well DNA strand breaks. However, a synergistic effect was observed in cultures exposed to 500 μg/mL EO. In conclusion, EO at concentrations up to 300 μg/mL or doses up to 400 mg/kg are not mutagenic in leukocytes and in mice, but do have antioxidative and protective effects against the cytotoxicity and clastogenesis induced by H_2O_2.

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1. Introduction

The use of medicinal plant extracts has increased in the last decades in Brazil. Although little information is available on their potential health risks, studies of genotoxicity can help to evaluate the safety and effectiveness of herbal health products (Bast et al., 2002). It is important to determine the potential genetic hazards of compounds present in medicinal plants, together with their beneficial effects to the human body. However, biological data on the medicinal properties associated with plant extracts with pharmacological activities are relatively scarce, especially regarding mutagenic potential (Lohman et al., 2001). The world's populations in developing countries depend largely on plants for their primary health care, due to poverty and lack of access to modern medicines (Akerele, 1993; Cordell, 1995). According to World Health Organization (2002), about 80% of the population in developing countries rely on herbal medicines at least for their primary health care. Moreover, few plants have been scientifically assessed regarding their quality, safety and efficacy (Cavalcanti et al., 2008a). In spite of this, there have been few studies of the Brazilian medicinal flora aimed at examining potential health risks.

Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Smith (Zingiberaceae) is a perennial plant growing widely in the subtropical and tropical regions of the world. Many species of the genus *Alpinia* provide a variety of medicinal properties, such as *A. zerumbet* and *Alpinia purpuratal*. These species have been commercialized in the food and cosmetic industries. However, their greatest importance arises from the medicinal properties of their essential oils which have been used in folk medicine (Victório, 2011). Some studies have reported different pharmacological properties of the essential oil of *A. zerumbet*, such as antihypertensive (Lahlou et al., 2003), antinociceptive (de Araújo et al., 2005), anxiolytic (Satou et al., 2010), antipsychotic and antioxidant (de Araújo et al., 2011) attributes.

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http://dx.doi.org/10.1016/j.fct.2012.03.038

In Brazil, *A. zerumbet* is known popularly as "colônia," and it is traditionally used for the treatment of intestinal disorders and hypertensive cardiovascular disease, and as an antispasmodic and anti-inflammatory agent (Leal-Cardoso and Fonteles, 1999; Zoghbi et al., 1999; Bezerra et al., 2000), and it has been reported to possess antioxidant property (Elzaawely et al., 2007a,b). Although *Alpinia* is generally believed to be well-tolerated, safety has not been well studied. Currently, there is not enough available scientific evidence for or against the use of *Alpinia* for any indication.

Due to the fact that *A. zerumbet* medicinal users employ teas and infusions prepared from its leaves (Leal-Cardoso and Fonteles, 1999), the aim of the current study was to further evaluate the genotoxic and mutagenic effects of the essential oil (EO) of *A. zerumbet* on peripheral blood leukocytes (PBLs) *in vitro* using the alkaline single-cell gel electrophoresis test (comet assay), chromosomal aberrations (CAs) test, and the cytokinesis-block micronucleus (MN) assay, as well as on mouse bone marrow and PBLs *in vivo* using the MN and comet tests. Furthermore, we evaluated the antioxidant potential of EO of *A. zerumbet* by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging assay in order to correlate it with chemopreventive effects (antimutagenesis) against oxidative damage induced by H_2O_2.

2. Materials and methods

2.1. Plant material

The leaves of *A. zerumbet* were collected in the municipality of Maranguape (3°59.264′S; 38°42.591′W) in Ceará State (Northeastern Brazil) during January 2008. The plant was identified by Drs. Edson Paula Nunes and Peres Martins (Department of Biology, Federal University of Ceará, Fortaleza, Brazil), and a voucher specimen (ICN: 41041) was deposited at Herbarium Prisco Bezerra (Federal University of Ceará).

2.1.1. Extraction

Fresh leaves of *A. zerumbet* (500 g) were cut into small pieces and submitted to hydrodistillation in a Clevenger-type glass apparatus during 2 h, affording a yellowish oil. The obtained oil was dried over anhydrous sodium sulfate, filtered and kept under refrigeration until the GC–MS and GC–FID analysis. The oil yield was calculated as 0.3% based on the fresh weight of the plant material (w/w).

2.1.2. Gas chromatography–mass spectrometry (GC–MS)

The GC–MS analysis was carried out on a Shimadzu QP5050 instrument equipped with a non-polar OV-5 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness), utilizing helium as carrier gas and flow rate of 1 mL/min, with split ratio of 1:48. The injector temperature and detector temperature were set at 250 and 280 °C, respectively. The oven temperature was programmed to increase from 40 to 180 °C at 4 °C/min, and afterwards to 280 °C at 20 °C/min, which was kept for 7 min. Mass spectra were recorded in a range of mass-to-charge ratio (m/z) between 30 and 450. The relative content of oil constituents was determined by peak area normalization and expressed as percentage. The volatile components were identified by comparison of their 70 eV mass spectra with those provided by a spectrometer database (Wiley L-built library) as well as comparing the fragmentation patterns with those reported in the literature (Adams, 2001).

2.1.3. Gas chromatography–flame ionization detector (GC–FID)

The GC–FID analysis was carried out on a Shimadzu GC 2010 Plus instrument equipped with a non-polar CP-Sil-8 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness), utilizing hydrogen as carrier gas, flow rate of 1.5 mL/min, with split ratio 1:30. The injector temperature and detector temperature were set at 250 and 260 °C, respectively. The oven temperature was programmed from 70 to 180 °C at 4 °C/min, afterwards it was raised to 250 °C at 10 °C/min, which was kept for 7 min. The relative content of oil constituents was determined by the peak area normalization and expressed as percentage. The volatile components were identified by comparison of the Kovats retention indices determined from the injection of a homologous series of *n*-alkanes (C_7–C_{30}) and by means of eight authentic analytical standards (β-pinene, α-terpinene, *p*-cymene, 1,8-cineol, terpin-4-ol, α-terpineol, caryophyllene, caryophyllene oxide) run in the same chromatographic conditions applied to sample.

2.2. Chemicals

Fetal bovine serum (FBS), phytohemagglutinin, RPMI 1640 medium, trypsin–EDTA, glutamine, penicillin and streptomycin were purchased from GIBCO® (Invitrogen, Carlsbad, CA, USA). Low-melting point agarose and agarose were obtained from Invitrogen (Carlsbad, CA, USA). Formamidopyrimidine DNA-glycosylase (FPG) was obtained from NewEngland BioLabs (USA). Colchicine, cytochalasin-B, methylmethanesulfonate (MMS), reduced glutathione (GSH), NADPH, glutathione reductase, 5,5′-dithionitrobenzoic acid (DTNB), DPPH, mixture of *n*-alkanes and analytical standards used in the GC–FID analysis were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Hydrogen peroxide was obtained from Vetec (Brazil). Cyclophosphamide was from ASTA MEDICA (Brazil). All other chemicals and reagents used were of analytical grade.

2.3. Peripheral blood leukocyte (PBL) isolation

Heparinized blood was collected from healthy, non-smoker donors who had not taken any medication for at least 15 days prior to sampling and who had no history of recent exposure to potentially genotoxic substances (i.e., pesticides, drugs, alcohol, tobacco) or ionizing radiation (i.e., X-rays). PBLs were isolated by the standard method of density-gradient centrifugation over Histopaque-1077. Cells were washed and resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C under 5% CO_2. Phytohemagglutinin (2.5%) was added at the beginning of culture. After 24 h of culture, cells were treated with the test substances.

2.4. Inhibition of PBL proliferation (Alamar Blue test)

The Alamar Blue test was performed with PBLs (1×10^6 cells/mL) after 48 h exposure to the test substances. The sample (50–500 μg/mL) dissolved in saline was added to each well, and the cells were incubated for 48 h. Control groups received the same amount of saline. Twenty-four hours before the end of the incubation, 10 μL of stock solution (0.312 mg/mL) of Alamar Blue (Resazurin, Sigma–Aldrich Co) were added to each well. The absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter®) and the drug effect was quantified as the percentage of control absorbance at 570 and 595 nm. The absorbance of Alamar Blue in culture medium is measured at a higher wavelength and a lower wavelength. The absorbance of the medium is also measured at the higher and lower wavelengths. The absorbance of the medium alone is subtracted from the absorbance of medium plus Alamar Blue at the higher wavelength. This value is called AO_{HW}. The absorbance of the medium alone is subtracted from the absorbance of medium plus Alamar Blue at the lower wavelength. This value is called AO_{LW}. A correction factor R_0 can be calculated from AO_{HW} and AO_{LW}, where $R_0 = AO_{LW}/AO_{HW}$. The percent Alamar Blue reduced by viable cells was expressed as follows: % reduced = $A_{LW} - (A_{HW} \times R_0) \times 100$ (Ahmed et al., 1994).

2.5. DPPH radical-scavenging assay

The free radical scavenging activity of test substances was measured using DPPH by the method of Blois (1958). A solution of DPPH (0.1 mM) in ethanol was prepared and added to various quantities of EO (50, 100, and 300 μg/mL) directly in wells of a multi-well plate. After 30 min, absorbance was measured at 517 nm. Ascorbic acid (150 μM) was used as the reference. All tests were performed in triplicate. DPPH radical-scavenging capacities (%) of test substances were calculated using the following equation:

$$\%\text{Scavenging} = \frac{\text{control absorbance-sample absorbance}}{\text{control absorbance}} \times 100$$

2.6. PBL treatments

For conventional genotoxic and mutagenic experiments, PBLs (5×10^5 cells/mL) were treated with increasing concentrations (50–500 μg/mL) of EO dissolved in saline (0.9%) or MMS (4×10^{-5} M) dissolved in DMSO (0.1%), without FBS, for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2.

In addition, EO of *A. zerumbet* was tested for its antioxidant potential in exerting an antimutagenic effect. The cytokinesis-block MN test and alkaline version of the comet assay were carried out in PBLs (0.5×10^6 cells/mL) exposed to 150 μM H_2O_2 for 1 h, along with pre-, co-, or post-treatment with EO at concentrations ranging from 50 to 500 μg/mL (without FBS), in order to correlate the possible mechanism of modulation (intra- and extracellular reactions) and effect on DNA repair. In the pre-treatment protocol, EO-treated cells (3 h) were washed with PBS, pH 7.4, and exposed to H_2O_2 for 1 h. In co-treatment protocol, PBLs were treated, at same time, with EO and H_2O_2 during 1 h. In the post-treatment protocol, H_2O_2-exposed PBLs (1 h) were washed with PBS before being treated with different EO concentrations for 3 h. In these sets of experiments, cell viability and apoptosis induction were also monitored.

2.7. Measurement of intracellular GSH content

After H_2O_2 (150 μM for 1 h) challenge (pre-, co-, and post-treatment protocols described above), the GSH content was determined by a spectrophotometric assay based on the formation of 5-thio-2-nitrobenzoate (TNB) from DTNB, according to Akerboom and Sies (1981) with minor modification. Briefly, treated (100 and 300 μg/mL EO) and untreated PBLs (1.5×10^6 cells/mL) were washed with ice-cold PBS, resuspended in 0.1 M sodium phosphate-5 mM EDTA, pH 8.0, and sonicated to obtain the cell homogenate. An equal volume of 2 M $HClO_4$–4 mM EDTA was added to the cell extract, and the precipitated proteins were pelleted by centrifugation at 8000*g* for 15 min at 4 °C. The supernatant was neutralized with 2 M KOH, and the insoluble residue was removed by centrifugation under the same conditions. For spectrophotometric determination, 910 μL of the cell extract supernatant or of a standard GSH solution, in the same phosphate–EDTA buffer, were mixed with 50 μL of 4 mg/mL NADPH in 0.5% (w/v) $NaHCO_3$, 20 μL of 6 U/mL glutathione reductase in phosphate–EDTA buffer, and 20 μL of 1.5 mg/mL DTNB in 0.5% $NaHCO_3$. The increase in absorbance was measured at 412 nm. The results were normalized by protein content (Lowry et al., 1951), and were expressed as μg/mg protein.

2.8. Lipid peroxidation–TBARS assay

After H_2O_2 (150 μM for 1 h) challenge (pre-, co-, and post-treatment protocols described above), the extent that test samples induced lipid peroxidation was determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product formed by lipid peroxidation (Draper and Hadely, 1990). The assays were performed according to Salgo and Pryor (1996), with minor modifications. Cells were incubated with the test samples for 3 h and then lysed with 15 mM Tris–HCl for 1 h. Two milliliters of trichloroacetic acid (0.4 mg/mL) and HCl (0.25 M) were added to the lysate, which was then incubated with 6.7 mg/mL TBA for 15 min at 100 °C. The mixture was centrifuged at 750*g* for 10 min. As TBA reacts with other products of lipid peroxidation in addition to MDA, results are expressed in terms of thiobarbituric reactive species (TBARS), which are determined by absorbance at 532 nm. Hydrolyzed 1,1,3,3-tetramethoxypropane was used as the standard. The results were normalized by protein content (Lowry et al., 1951).

2.9. Cell viability and morphological characterization of apoptotic PBLs

Cell viability was determined by the trypan blue dye exclusion test. After treatment, trypan blue-excluding cells in samples taken from cultures were counted in a Neubauer chamber. The percentage of viable cells was then calculated (Cavalcanti et al., 2008b). Apoptotic cells were determined after each treatment by the acridine orange (AO)/ethidium bromide (EB) staining assay: 25 μL of the cell suspension were mixed with 1 μL of the staining solution (100 μg/mL AO + 100 μg/mL EB in PBS) and spread on a slide, where 300 cells were counted per data point. The percentage of apoptotic cells was then calculated (McGahon et al., 1995).

2.10. In vitro alkaline comet assay

The alkaline comet assay was performed as described by Singh et al. (1988) with minor modifications (Hartmann and Speit, 1997), and following the recommendations of the International Workshop on Genotoxicity Test Procedures (Tice et al., 2000). At the end of the treatment, cells were washed with ice-cold PBS, detached with 100 μL trypsin (0.15%) and resuspended in complete RPMI medium. Next, 20 μL of cell suspension (~10^6 cells/mL) were mixed with 100 μL of 0.75% low melting point agarose and immediately spread onto a glass microscope slide precoated with a layer of 1% normal melting point agarose. The agarose was allowed to set at 4 °C for 5 min. The slides were incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4 °C for a minimum of 1 h to remove cellular proteins, leaving the DNA as "nucleoids." After the lysis procedure, the slides were placed on a horizontal electrophoresis unit. The unit was filled with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13.0) to cover the slides for 20 min at 4 °C to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was carried out for 20 min at 25 V and 300 mA (0.86 V/cm).

After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with ethidium bromide (20 μg/mL) and analyzed using a fluorescence microscope. All the above steps were conducted under yellow light or in the dark to prevent additional DNA damage. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed for each concentration of test substance. Cells were scored visually and assigned to one of five classes, according to tail size (from undamaged-0, to maximally damaged-4), and a damage index value was calculated for each sample of cells. Damage index thus ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) (Collins, 2004). The frequency of tailed cells, a DNA damage frequency indicator, was calculated based on the number of cells with tails (DNA strand breaks) vs. those without.

2.11. Measurement of oxidized purines and of intracellular reactive oxygen species (ROS)

Oxidized purines and ROS production were estimated by the alkaline comet assay (as described above) and by using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) as fluorescence probe, respectively, after H_2O_2 (150 μM for 1 h) challenge (pre-, co-, and post-treatment protocols described above).

For comet assay, briefly, the slides were removed from the lysis solution, and washed three times in enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM Na_2-EDTA, 0.2 mg/mL BSA, pH 8.0), drained, and incubated with 70 μL FPG (30 min 37 °C). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were visually analyzed per group. The amount of oxidized purines (FPG-sensitive sites) was then determined by subtracting the amount of strand breaks (samples incubated with buffer alone) to the total amount of breaks obtained after incubation with FPG according to da Silva Júnior et al. (2011).

For ROS detection, at the end of the treatments, cells were incubated with 20 μM H2-DCFDA for 30 min in the dark at 37 °C. Then, cells were then harvested, washed and re-suspended in PBS and analyzed immediately via flow cytometry (Guava EasyCyte Mini; Guava Technologies, Inc., Hayward, CA, USA). H_2DCFDA diffuses through the cell membrane readily and is hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescin (DCFH), which is then rapidly oxidized to highly fluorescent DCF (2′,7′-dichlorofluorescein) by a broad range of intracellular oxidative stresses other than H_2O_2 (Hempel et al., 1999). DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly (LeBel et al., 1992).

2.12. Chromosomal aberrations (CAs) test

After the end of treatment, PBLs were washed with ice-cold PBS and recultivated in complete RPMI medium for 48 h. Colchicine (0.0016%) was added 2 h before fixation (72 h). Chromosomes were prepared according to standard procedures (Moorhead et al., 1960). Hypotonic treatment with KCl (0.75 M, 37 °C) was applied for 15 min. The cells were fixed with methanol/acetic acid (3:1), and the fixative solution was changed twice. Air-dried slides were stained with Giemsa (5%, pH 6.8) for 10 min and scored for CAs according to Savage (1976). Gap cells were also recorded, but not considered for the evaluation of mutagenicity. MMS (4×10^{-5} M) was used as the positive control. Only well-spread metaphases were examined. One hundred and fifty metaphases per culture were analyzed for the presence of CAs. The mitotic index was determined for 2000 cells and given as the number of mitoses per 100 cells (%) (Arni and Hertner, 1997).

2.13. Cytokinesis-block micronucleus (MN) assay

After treatment, PBLs were washed twice with medium, and cytochalasin-B (3 μg/mL) was added to the cultures at 44 h post-initiation, as described by Fenech (2000). Cells were harvested 72 h after the treatment starting point, resuspended in 75 mM KCl, kept at 4 °C for 3 min (mild hypotonic treatment), and fixed with cold methanol/acetic acid (3:1) solution. This fixation step was repeated twice, and finally, the cells were resuspended in a small volume of methanol/acetic acid and dropped onto clean slides. Slides were stained with 10% Giemsa (pH 6.8) for 6 min, mounted and coded prior to microscopic analysis. Micronuclei were counted in 2000 binucleated cells (BNC) with well-preserved cytoplasm. The identification of MN was carried out according to Fenech (2000).

2.14. Animals and experimental design

Male and female Swiss mice, 7 weeks old, weighing approximately 25 g, were obtained from the Federal University of Ceará animal house and submitted to 1 week of acclimatization. The animals were maintained in an experimental room under controlled conditions of temperature (22 ± 2 °C), humidity (~60 °C) and a 12-h light/dark cycle, with *ad libitum* access to food and water. The assays were performed using 5 animals/group. EO at 400 mg/kg was administered by the oral route. The negative and positive control groups received orally saline and cyclophosphamide (25 mg/kg), respectively. All treatments consisted of a single oral dose. Bone marrow samples were obtained from the mice at 24 and 48 h after the administration of the test substances. All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

2.14.1. Mouse bone marrow micronucleus (MN) test

Before the animals were sacrificed, both femora were dissected and the marrow cells were flushed out with 1 mL of fetal bovine serum and pipetted up and down several times. The cell suspension was centrifuged at 1000 rpm for 5 min, the supernatant was removed, and the cell pellet was resuspended and placed on a clean glass slide. The preparations were dried overnight, covered with concentrated Leishman's stain for 3 min and counter-stained with diluted Leishman's stain (1:6) for 15 min. The slides were scored under a light microscope at 40× magnification. The percentage of micronucleated cells was determined relative to a differential blind count of 2000 polychromatic erythrocytes (PCEs) per animal (Ramos et al., 2008).

2.14.2. In vivo alkaline comet assay

For the *in vivo* comet assay, the experimental design was the same as for the MN test in mice. Peripheral blood was collected in heparinized capillary tubes and kept on ice until use. The comet assay was conducted under alkaline conditions (pH > 13). The experiments were carried out according to the protocol of Hartmann et al. (2003). Five animals were used per experimental group, and three slides were prepared from each blood sample. The experimental procedure and analysis of the cells were performed as described above.

2.15. Statistical analysis

All experiments were performed in triplicate in three independent experiments. All statistical analyses were carried out using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). For the analysis of DPPH radical-scavenging capacity, cell viability and proliferation, apoptosis induction, and comet and cytokinesis-block MN assays, data of normal distribution were compared by analysis of variance (ANOVA) followed by Tukey's test. For induction of *in vitro* CAs data of normal distribution were compared by ANOVA followed by the paired Student's *t*-test. Data of normal distribution from the *in vivo* MN test were compared by ANOVA followed by Dunnett's test.

3. Results

3.1. GC/MS and GC–FID analysis analysis of essential oil of A. zerumbet

The GC/MS and GC–FID analysis of the hydrodistilled EO from *A. zerumbet* leaves permitted the detection and identification of sixteen constituents, 14 of which were monoterpenes (98.23%), along with two sesquiterpenes (1.77%) (Table 1). 1,8-Cineol (22.40%) and *p*-cymene (18.91%), terpinen-4-ol (17.32%) were the major compounds of the *A. zerumbet* oil. These data are in agreement with those reported in the literature (Lahlou et al., 2003; Murakami et al., 2009; Victório et al., 2010).

3.2. DPPH radical-scavenging activity and variation on intracellular GSH pool after A. zerumbet's essential oil exposure

1,1-Diphenyl-2-picryl-hydrazyl radical (DPPH·), an organic free radical, was used to study the free radical-scavenging capacity of EO of *A. zerumbet*. DPPH· is considered to be a model for the lipophilic radical. A chain reaction in lipophilic radicals is initiated by lipid autoxidation (Blois, 1958; Kumar et al., 2005). The positive DPPH test suggests that EO contains free radical scavengers. The scavenging effects of EO on DPPH· are illustrated in Fig. 1. EO had significant ($p < 0.05$) scavenging effects on DPPH· which increased with increasing concentration in the 50–300 μg/mL range. The scavenging capacity of control (saline) was zero, while EO at the lowest concentrations (50 and 100 μg/mL) scavenged DPPH· by about 32.25 ± 4.78% at 50 μg/mL and 65.14 ± 3.78% at 100 μg/mL. At the highest concentration of EO, the scavenging capacity was similar to that of vitamin C (ascorbic acid) at 150 μM. The DPPH radical-scavenging capacity (%) for EO at 300 μg/mL was 80.15 ± 2.08%, and for vitamin C 85.33 ± 6.17%.

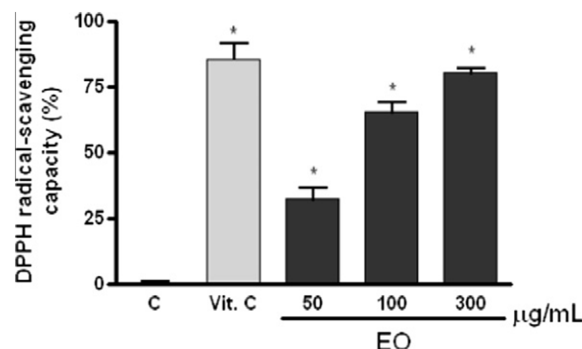

Fig. 1. DPPH radical-scavenging capacity (%) of essential oil (EO) of *A. zerumbet*. Negative control (C) was treated with the vehicle (saline), used for diluting the test substances, and 150 μM vitamin C (vit. C) was used as reference. Bars represent the mean ± SEM of three independent experiments. *$p < 0.05$; vs. control (saline) – ANOVA followed by Tukey's test.

As seen in Tables 2 and 3, EO (100 and 300 μg/mL) did not reduce intracellular GSH levels, and did not induce an increase in the oxidative stress markers after 3-h treatment, while H_2O_2 caused a statistically significant decrease in GSH content, and provoked an increase on intracellular ROS generation and lipid oxidation products (MDA formation), as well an increase in the purine bases oxidation after 1 h incubation. After H_2O_2 challenge, only co-treatment preserved the intracellular GSH levels at control values, and also prevented the ROS formation, lipid peroxidation, and the oxidation of nucleotidic bases. EO pre-incubation or post-incubation did not prevent the decrease in GSH content (Table 2) and the others events of oxidative stress (Table 3).

3.3. In vitro cytotoxic, genotoxic, and mutagenic effects of essential oil of A. zerumbet in PBLs

After 48 h exposure, the Alamar Blue assay and trypan blue dye exclusion test showed that EO of *A. zerumbet* did not elicit any significant antiproliferative or toxic effects on PBLs cultures at concentrations up to 300 μg/mL. However, cultures exposed to the highest concentration (500 μg/mL) of EO showed a decrease in

Table 1
Chemical composition of the essential oil (EO) from the leaves of *A. zerumbet*.

Volatile components	IK[a]	Relative area (%)[b]
α-Thujene	931	3.84
α-Pinene	940	2.12
Sabinene	978	9.90
β-Pinene	983	3.60
Myrcene	992	0.80
α-Terpinene	1021	2.50
p-Cymene	1029	18.91
Limonene	1034	2.42
1,8-Cineol	1037	22.40
γ-Terpinene	1063	11.42
Terpinolene	1092	1.18
Linalool	1101	1.04
Terpinen-4-ol	1182	17.32
α-Terpineol	1193	0.78
β-Caryophyllene	1424	1.11
Caryophyllene oxide	1587	0.66

[a] IK-Kovats retention indices calculated from a homologous series of *n*-alkanes (C_7–C_{30}) analyzed on a CP-Sil-8 column.
[b] Relative area percentage determined by GC–FID.

Table 2
Effects of essential oil (EO) on PBLs intracellular GSH after H_2O_2 challenge.

Compounds	Treatment	GSH (μg/mg protein)
Saline[a]	0.9%	3.71 ± 0.23
H_2O_2[b]	150 μM	1.46 ± 0.56**
EO	100 μg/mL	3.98 ± 0.35*
	300 μg/mL	3.55 ± 0.21*
Pre-exposure	100 μg/mL	1.40 ± 0.61**
	300 μg/mL	1.66 ± 011**
Co-exposure	100 μg/mL	3.37 ± 0.15*
	300 μg/mL	3.21 ± 0.15*
Post-exposure	100 μg/mL	2.04 ± 0.17**
	300 μg/mL	1.93 ± 0.33**

[a] Negative control used for diluting the essential oil.
[b] Positive control.
* $p < 0.05$ Compared to positive control (H_2O_2).
** $p < 0.05$ compared to saline group by ANOVA followed by Tukey test. Data are presented as means ± SEM for three independent experiments in triplicate.

Table 3
Effects of essential oil (EO) on PBLs lipid peroxidation, oxidation of purine bases, and intracellular ROS after H_2O_2 challenge.

Compounds	Treatment	MDA equivalents (nmol//mg protein)	FPG-sensitive sites (damage index)	ROS (%)
Saline[a]	0.9%	3.69 ± 0.01	8.35 ± 2.15	1.77 ± 0.15
H_2O_2[b]	150 μM	27.14 ± 2.45**	231.08 ± 5.16**	83.63 ± 3.17**
EO	100 μg/mL	2.89 ± 1.08	6.89 ± 1.25	2.15 ± 0.01
	300 μg/mL	2.51 ± 0.17	7.03 ± 0.56	1.85 ± 0.10
Pre-exposure	100 μg/mL	19.75 ± 1.18**	218.39 ± 4.23**	69.24 ± 1.25**
	300 μg/mL	23.64 ± 3.15**	225.23 ± 2.45**	73.18 ± 5.15**
Co-exposure	100 μg/mL	3.21 ± 0.11*	4.82 ± 0.15*	2.13 ± 0.10*
	300 μg/mL	3.37 ± 0.01*	6.33 ± 0.21*	1.98 ± 0.21*
Post-exposure	100 μg/mL	24.61 ± 0.75**	198.27 ± 7.15**	76.21 ± 2.45**
	300 μg/mL	22.37 ± 1.18**	202.63 ± 3.25**	70.43 ± 1.25**

[a] Negative control used for diluting the essential oil.
[b] Positive control.
* $p < 0.05$ Compared to positive control (H_2O_2).
** $p < 0.05$ Compared to saline group by ANOVA followed by Tukey test. Data are presented as means ± SEM for three independent experiments in triplicate.

the proliferation rate and viability of PBLs (Fig. 2). The preceding findings correlated well with the results of genotoxic (comet assay) and mutagenic (MN and CAs) assays. EO was not able to increase DNA strand breaks (Fig. 3) and the frequency of aberrant PBLs (Table 4) and micronucleated PBLs, and did not reduce the cytokinesis-block proliferation index (Table 5), at concentrations ranging from 50 to 300 μg/mL. On the other hand, at the highest concentration, EO caused a significant increase in the cell DNA damage index and in the frequencies of tailed, aberrant, and micronucleated PBLs, and decrease in the cytokinesis-block proliferation index. In CAs evaluation, the most frequent alterations found were chromosome and chromatid breaks and gaps. Rearrangements such as dicentric and ring chromosomes and triradial figures were not observed. As expected, the exposure of PBLs to the alkylating agent MMS resulted in a significant increase in DNA strand breaks (31.24-fold) as well as in the number of aberrant cells (16.81-fold) and micronucleated cells (17.50-fold) when compared to the negative control (saline).

3.4. Effects on H_2O_2 challenge

The ability of non-toxic (50–300 μg/mL) and toxic concentrations (500 μg/mL) of EO to reduce H_2O_2 toxicity as measured by the trypan blue assay and AO/EB DNA-binding fluorescent dye-based approach was determined. Figs. 4 and 5 show that H_2O_2 (150 μM) cytotoxicity (decreased viability and apoptosis induction) was significantly attenuated by non-toxic concentrations of EO in co-, and post-treatment protocols, respectively. However, EO at 500 μg/mL significantly enhanced H_2O_2-induced cytotoxic effects ($p < 0.05$). The effect of the test substances on H_2O_2-induced DNA damage is shown in Tables 6 and 7, along with the mutagenic and antimutagenic effects of EO determined by the cytokinesis-block MN assay after co-, and post-exposure, respectively. As expected, PBLs treated with 150 μM H_2O_2 exhibited severe DNA damage, as demonstrated by the increase in DNA damage index. When PBLs were co- or post-exposed to non-toxic concentrations of EO, the levels of DNA strand breaks were significantly reduced ($p < 0.05$). Also, permanent DNA damage was reduced at concentrations up to 300 μg/mL, as shown by the concentration-dependent decrease in micronucleus rate. In contrast, a synergistic effect was observed in PBLs co-, and post-treated at the highest concentration of EO. These results obtained by co- or post-exposure protocols indicated that treatment with EO of *A. zerumbet* at non-toxic concentrations (50–300 μg/mL) protected PBLs against the mutagenic oxidative effects of H_2O_2. On the other hand, pre-treatment (EO) did not protect PBLs against the cytotoxic (Fig. 6A) and clastogenic effects of H_2O_2 (Figs. 6B and C).

3.5. Essential oil of A. zerumbet lack genotoxic and mutagenic effects in mice

In the comet assay, peripheral blood was collected 24 and 48 h after the administration of the test substances. The results of the genotoxicity test in mouse peripheral blood cells after EO

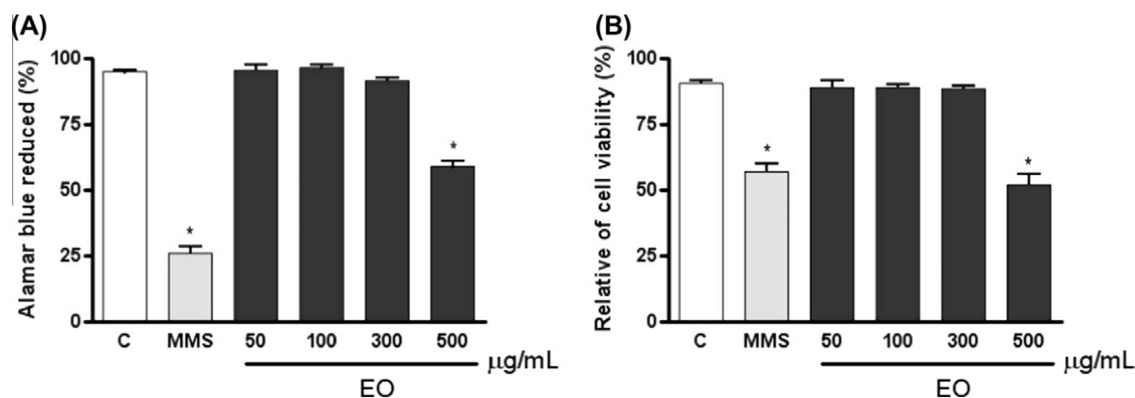

Fig. 2. Effects of essential oil (EO) of *A. zerumbet* on PBLs proliferation by Alamar Blue test (panel A), and on cell viability using trypan blue dye exclusion after 48 h of incubation (panel B). Negative control (C) was treated with the vehicle (saline), used for diluting the test substances, and 4×10^{-5} M methylmethanesulfonate (MMS) was used as the positive control. Bars represent the mean ± S.E.M. of three independent experiments. *$p < 0.05$; vs. control (saline) – ANOVA followed by Tukey's test.

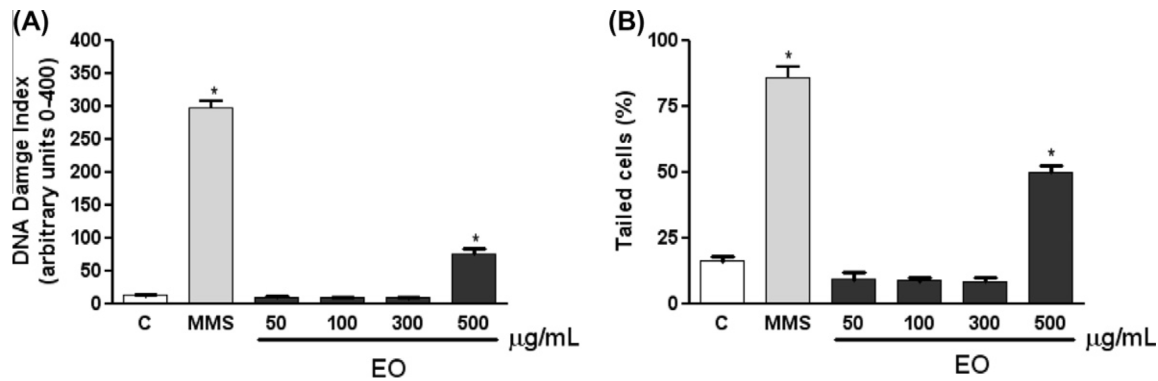

Fig. 3. Effects of essential oil (EO) of *A. zerumbet* on PBL DNA damage index (panel A) and percentage of tailed cells (panel B) after 24 h of incubation, determined by the alkaline comet assay. Negative control (C) was treated with the vehicle (saline), used for diluting the test substances, and 4×10^{-5} M methylmethanesulfonate (MMS) was used as the positive control. Bars represent the mean ± SEM of three independent experiments. *$p < 0.05$; vs. control (saline) – ANOVA followed by Tukey's test.

Table 4
Mitotic index, frequency of chromosomal aberrations, and numeric changes in cultured human leukocytes after *A. zerumbet* essential oil (EO) exposure.

Substance	Treatment	Exp.	Mitotic index[c]		Number of aberrations[d]				Aberrant cells[e]	
			%	Mean ± SD	G	R	P	E	%	Mean ± SD
MMS[a]	4×10^{-5} M	1	1.8	1.40 ± 0.36*	1	10	0	0	6.6	7.23 ± 2.41*
		2	1.1		4	8	0	0	5.2	
		3	1.3		1	15	0	0	9.9	
Saline[b]	0.9%	1	6.4	6.46 ± 0.40	0	0	0	1	0.0	0.20 ± 0.34
		2	6.1		0	1	0	0	0.6	
		3	6.9		2	0	0	0	0.0	
EO	50 µg/mL	1	6.8	6.60 ± 0.26	1	0	0	0	0.0	0.0
		2	6.3		5	0	0	0	0.0	
		3	6.7		1	0	0	0	0.0	
	100 µg/mL	1	6.0	6.03 ± 0.35	0	0	0	1	0.0	0.40 ± 0.69
		2	5.7		0	0	0	0	0.0	
		3	6.4		1	2	0	0	1.2	
	300 µg/mL	1	5.5	5.66 ± 0.47	3	0	0	0	0.0	0.20 ± 0.34
		2	5.3		1	0	0	1	0.0	
		3	6.2		0	1	0	1	0.6	
	500 µg/mL	1	4.3	4.10 ± 0.20*	1	5	0	3	3.3	3.23 ± 1.90*
		2	4.1		0	2	0	2	1.3	
		3	3.9		3	7	0	0	5.1	

[a] Positive control.
[b] Vehicle.
[c] Frequency per experiment, mean and standard deviation, in 2000 cells.
[d] Number of aberrations per 150 metaphases analysed.
[e] Frequency per experiment, mean and standard deviation of aberrant cells excluding gaps; G, gaps (chromosome and chromatid); R, ruptures (chromosome and chromatid); P, polyploid cells; E, endoreduplication.
* Data significant in relation to control group (vehicle) at $p < 0.001$/ANOVA followed by Student's *t*-test.

treatment are shown in Fig. 7. Analysis within the 24 and 48-h groups did not show any significant increase in the DNA damage index. The levels of DNA strand breaks in groups sacrificed at 24 and 48 h after treatment were similar to control levels (saline). Table 8 showed that there was no significant increase in the incidence of micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow of male and female mice treated with EO at 400 mg/kg. Moreover, in all sets of experiments, no statistical differences in DNA damage levels and MNPCEs frequencies between sexes were observed in cells collected at different times (24 and 48 h) after EO administration.

Table 5
Effects of essential oil (EO) of *A. zerumbet* on PBLs micronucleated cell frequency in the micronucleus (MN) test.

Samples	Treatment	MN per 2000 BNC[c]	% BNC
Saline[a]	0.9%	4.18 ± 2.45	87.14 ± 0.75
MMS[b]	4×0^{-5} µM	73.16 ± 1.12*	43.67 ± 0.96*
EO	50 µg/mL	6.41 ± 0.86	90.17 ± 2.03
	100 µg/mL	5.50 ± 0.33	92.28 ± 0.75
	300 µg/mL	4.76 ± 1.16	89.64 ± 1.15
	500 µg/mL	21.44 ± 4.25*	51.23 ± 0.86*

[a] Vehicle used for diluting the test substances.
[b] Methylmethanesulfonate (MMS; positive control).
[c] MN frequency is expressed per 2000 binucleated cells (BNC).
* $p < 0.05$ Compared to control by ANOVA followed by Tukey's test. Data are presented as means ± SEM for three independent experiments in triplicate.

4. Discussion

Species of the genus *Alpinia* are extensively used for medicinal purposes in various parts of Asia and the Americas. Despite the popularity of *A. zerumbet* as an herbal remedy and the documented efficacy of its EO, the plant and its EO have received little scientific attention (de Araújo et al., 2005). The use of plants for the treatment of diseases continues to rise, although there are few studies

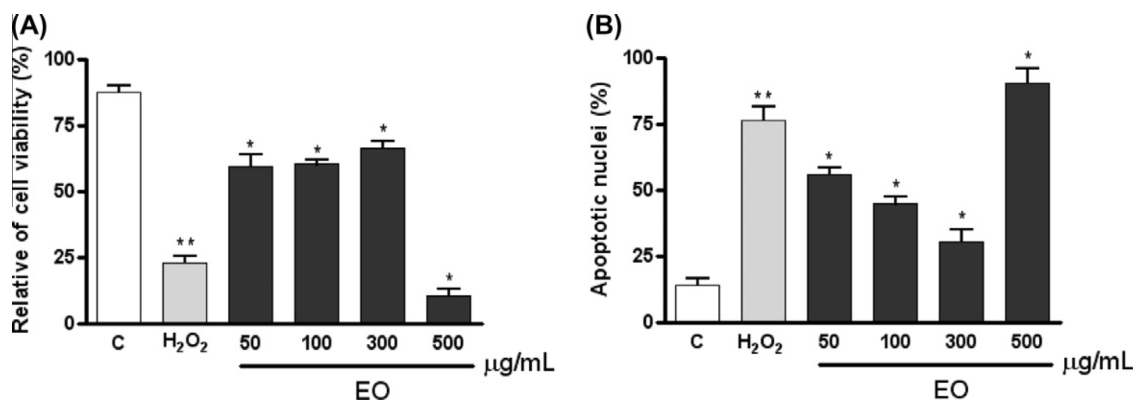

Fig. 4. Effects of co-treatment with essential oil (EO) of *A. zerumbet* on H_2O_2 (150 μM)-induced cytotoxicity. After H_2O_2 challenge, PBL viability and the induction of apoptotic cells were measured by the trypan blue dye exclusion test (panel A) and AO/EB staining (panel B), respectively. Negative control (C) was treated with the vehicle (saline), used for diluting the test substances. Bars represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; vs. positive control (H_2O_2); $^{**}p < 0.05$; vs. negative control (saline) – ANOVA followed by Tukey's test.

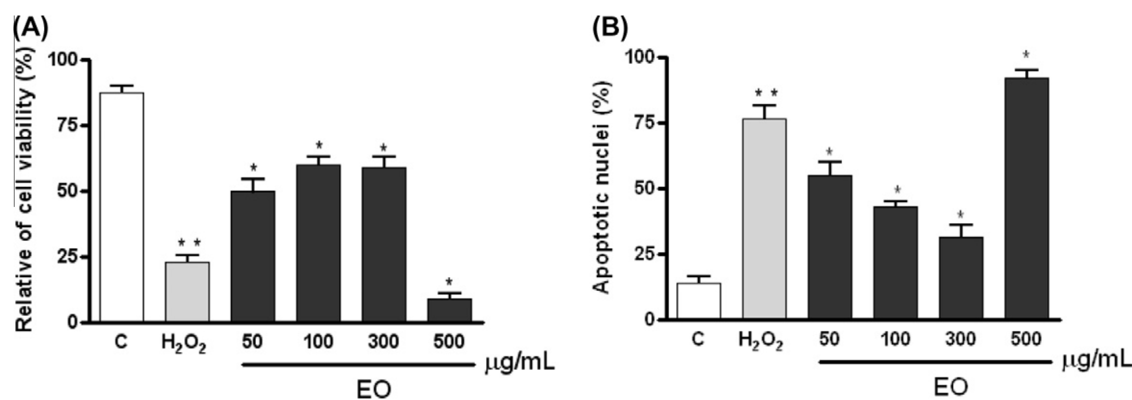

Fig. 5. Effects of post-treatment with essential oil (EO) of *A. zerumbet* on H_2O_2 (150 μM)-induced cytotoxicity. After H_2O_2 challenge, PBL viability and the induction of apoptotic cells were measured by trypan blue dye exclusion test (panel A) and AO/EB staining (panel B), respectively. Negative control (C) was treated with the vehicle (saline), used for diluting the test substances. Bars represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; vs. positive control (H_2O_2); $^{**}p < 0.05$; vs. negative control (saline) – ANOVA followed by Tukey's test.

providing proof of these effects. Previous studies have also indicated that some substances present in some medicinal plants are potentially toxic and carcinogenic (de Sá-Ferreira and Vargas, 1999), and it has also been reported that some traditional medicines may have a genotoxic potential (Sohni et al., 1994; Basaran et al., 1996; Romero-Jimenez et al., 2005; Cavalcanti et al., 2006; Demma et al., 2009). Assessment of the potential genotoxicity of traditional medicines is indeed an important issue, since damage to genetic material may lead to critical mutations and therefore to an increased risk of cancer and other diseases (Demma et al., 2009).

According to Faust et al. (2004), comet assay results generally agree with the findings of one or more cytogenetic assays such as chromosomal aberrations (CAs), micronucleus (MN) and sister chromatid exchange. Therefore, in the present study, to evaluate the magnitude of DNA damage, we used the alkaline version of the comet assay. This test is a sensitive method for detecting DNA strand breaks in individual cells (Collins, 2004). The present results indicate that EO of *A. zerumbet* did not exert genotoxic effects (DNA strand breaks) on human PBLs *in vitro* as well as peripheral blood cells of mice treated with non-toxic concentrations or dose. For mutagenic studies, we performed the *in vitro*

Table 6
Effects of co-treatment of essential oil (EO) of *A. zerumbet* on H_2O_2-induced chromosome breakage and clastogenesis in PBLs.

Samples	Treatment	Damage index	MN per 2000 BNC[b]
Saline[a]	0.9%	5.73 ± 0.25	2.05 ± 0.10
H_2O_2	150 μM	218 ± 5.15**	85.27 ± 3.25**
EO	50 μg/mL plus H_2O_2	107.14 ± 2.15*	65.42 ± 2.05*
	100 μg/mL plus H_2O_2	84.28 ± 0.10*	43.18 ± 0.20*
	300 μg/mL plus H_2O_2	76.31 ± 0.25*	21.71 ± 1.15*
	500 μg/mL plus H_2O_2	276.49 ± 6.17*	97.08 ± 4.75*

[a] Vehicle used for diluting the test substances.
[b] MN frequency is expressed per 2000 binucleated cells (BNC).
* $p < 0.05$ Compared To H_2O_2 group.
** $p < 0.05$ Compared to saline group by ANOVA followed by Tukey's test. Data are presented as means ± SEM for three independent experiments in triplicate.

Table 7
Effects of post-treatment of essential oil (EO) of *A. zerumbet* on H_2O_2-induced chromosome breakage and clastogenesis in PBLs.

Samples	Treatment	Damage index	MN per 2000 BNC[b]
Saline[a]	0.9%	5.73 ± 0.25	2.05 ± 0.10
H_2O_2	150 μM	218 ± 5.15**	85.27 ± 3.25**
EO	50 μg/mL plus H_2O_2	132.48 ± 0.15*	51.16 ± 0.11*
	100 μg/mL plus H_2O_2	111.55 ± 0.33*	37.63 ± 0.11*
	300 μg/mL plus H_2O_2	88.41 ± 0.11*	14.87 ± 0.33*
	500 μg/mL plus H_2O_2	239.73 ± 3.25*	102.14 ± 1.15*

[a] Vehicle used for diluting the test substances.
[b] MN frequency is expressed per 2000 binucleated cells (BNC).
* $p < 0.05$ Compared to H_2O_2 group.
** $p < 0.05$ Compared to saline group by ANOVA followed by Tukey's test. Data are presented as means ± SEM for three independent experiments in triplicate.

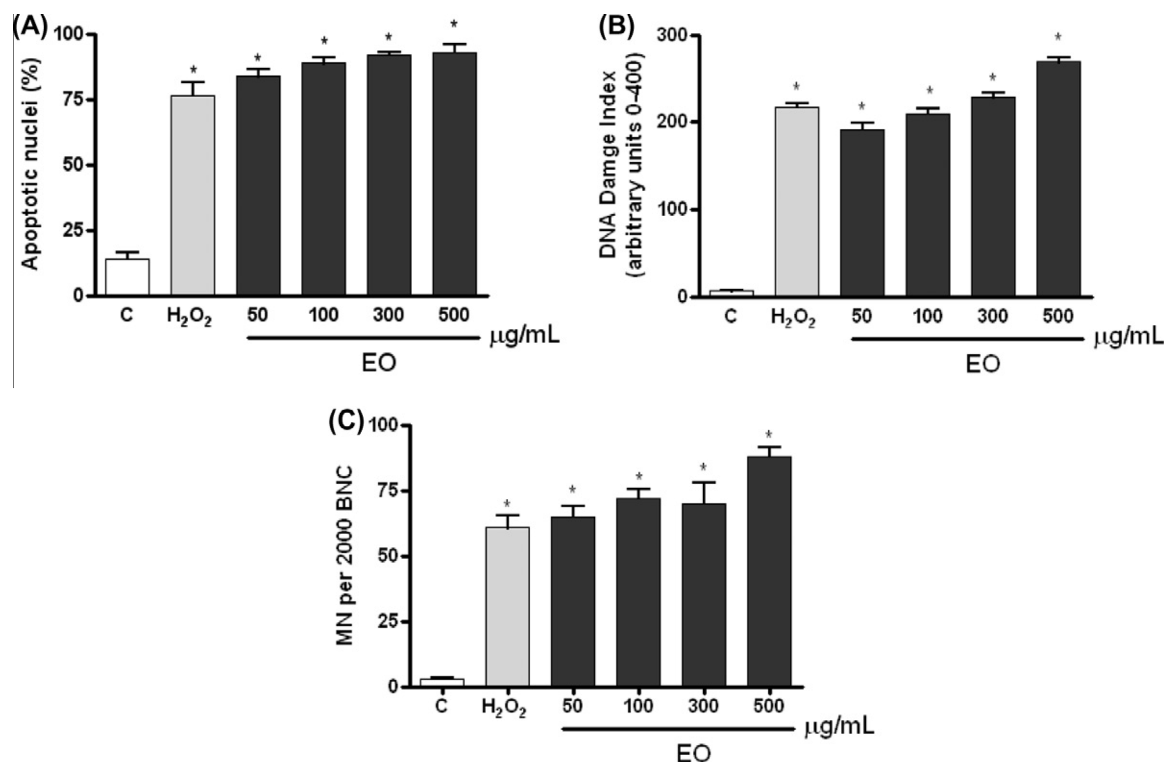

Fig. 6. Effects of pre-treatment with essential oil (EO) of *A. zerumbet* on H_2O_2 (150 μM)-induced cytotoxicity and oxidative DNA damage. After H_2O_2 challenge, the induction of apoptotic cells (panel A) was evaluated by AO/EB staining, and the protective effects of EO against clastogenic effects of H_2O_2 was assessed by the alkaline comet assay (panel B) and cytokinesis-block MN test (panel C). Negative control (C) was treated with the vehicle (saline), used for diluting the test substances. Bars represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; vs. control (saline) – ANOVA followed by Tukey's test.

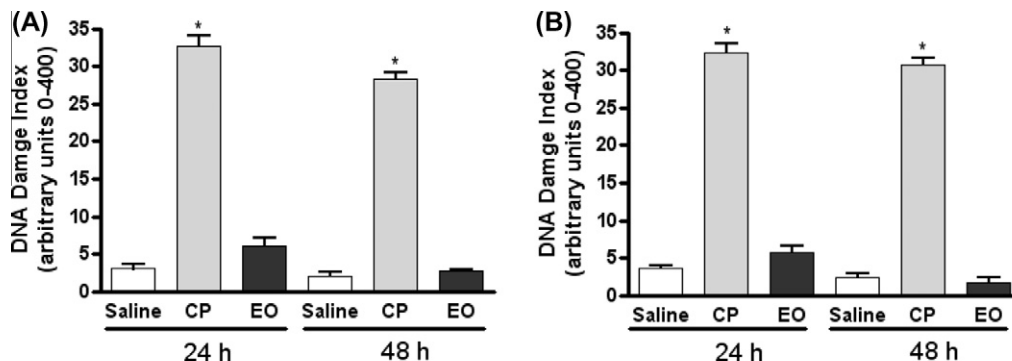

Fig. 7. DNA damage index in peripheral blood cells of mice treated with essential oil (EO) of *A. zerumbet* at 400 mg/kg as assessed by the alkaline comet assay after 24 and 48 h of exposure: male mice (panel A), and female mice (panel B). Saline, used for diluting the test substances, and 25 mg/kg cyclophosphamide (CP) were used as negative and positive controls, respectively. Bars represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; vs. control (saline) – ANOVA followed by Tukey's test.

CAs test as well the *in vitro* and *in vivo* MN assay, in which the latter is a very accurate and efficient tool to detect CAs as micronuclei (Cavalcanti et al., 2008b; Ramos et al., 2008). In both situations (*in vitro* and *in vivo* experiments), EO was not able to increase the frequency of aberrant and micronucleated PBLs or PCEs. The lack of *in vivo* mutagenic effect of EO of *A. zerumbet* corroborates the findings of Dias and Takahashi (1994), who showed that *A. nutans* Rosc (150–1200 mg/kg) was also devoid of mutagenic potential in a rodent model (*Rattus norvegicus*). Moreover, in PBL cultures exposed to EO of *A. zerumbet*, the proliferative rate (% BNC) was not affected by treatments at non-toxic concentrations (50–300 μg/mL), which agrees with the cytotoxicity data in the Alamar Blue, mitotic index, and cell viability (trypan blue dye exclusion) assays.

In contrast, the exposure of PBL cultures to the highest concentration (500 μg/mL) caused an increase in the levels of DNA damage and in the frequencies of cytogenetic abnormalities (CAs and MN). These DNA damage effects observed in PBL cultures treated with the highest concentration may be related to the toxic effects observed in our cytotoxicity and cell viability tests. It has been reported that besides primary DNA damage, secondary effects such as DNA strand-breaks indirectly induced as a consequence of cytotoxicity may also lead to an increase in DNA migration (Hartmann and Speit, 1997; Henderson et al., 1998; Hartmann et al., 2001).

Nowadays, cancer is one of the mortality factors in the world that occurs as a result of different causes, such as mutagenic and carcinogenic chemicals in the environment. The prevention of cancer and other related diseases can be achieved by avoiding expo-

Table 8
Effects of essential oil (EO) of *A. zerumbet* on micronucleus assay in bone marrow of male and female mice treated by a single oral dose and sampled 24 and 48 h after administration.

Sex	Substance	Treatment	Time (h)[c]	MNPCE[d]					
				Individual data	Mean ± SD				
Male	Saline[a]	0.9%	24	0	0	1	0	2	0.60 ± 0.89
			48	0	0	0	1	1	0.40 ± 0.54
	CP[b]	25 mg/kg	24	6	4	7	4	9	6.00 ± 2.12*
			48	5	7	4	9	7	6.40 ± 1.94*
	EO	400 mg/kg	24	1	1	0	1	1	0.80 ± 0.44
			48	0	0	0	1	0	0.20 ± 0.44
Female	Saline[a]	0.9%	24	0	1	0	0	1	0.40 ± 0.54
			48	0	0	0	1	1	0.40 ± 0.54
	CP[b]	25 mg/kg	24	7	7	9	5	7	7.00 ± 1.41*
			48	3	8	5	2	4	4.40 ± 2.30*
	EO	400 mg/kg	24	0	0	2	0	1	0.60 ± 0.89
			48	0	1	1	0	1	0.60 ± 0.54

[a] vehicle.
[b] CP (cyclophosphamide) as positive control.
[c] Exposure time.
[d] Number of micronucleated PCE (MNPCE) in 2000 PCE/animal and mean and standard deviation.
* Data significant in relation to control (vehicle) group at $p < 0.001$/ANOVA followed by Dunett's test.

sure to known mutagenic/carcinogenic agents, as well as by the consumption of protective factors or by chemoprevention, which involves the strengthening of physiological defense mechanisms through diet intervention or the use of drugs in the host organism (De Flora, 1998). Understanding the actions of antimutagenic agents can contribute to the prevention of tumors in individuals exposed to different mutagenic agents.

During normal cell metabolism, free radicals are produced at a high rate, but antioxidant defense systems or chemopreventive agents quench or minimize the production of free radicals and thereby protect cells from oxidative damage (i.e., DNA, proteins, plasma membrane). Many plant extracts have demonstrated potent cancer chemopreventive properties (Block, 1992; Ames and Gold, 1998; Lambert and Yang, 2003). Most of these extracts are known to exert their effects through antioxidant mechanisms, by either quenching reactive oxygen species (ROS), inhibiting lipid peroxidation or stimulating cellular antioxidant defenses (Park and Pezzuto, 2002; Valko et al., 2007).

Phytochemical studies with *A. zerumbet* and other *Alpinia* species revealed the presence a variety of phenolic compounds and other chemical constituents responsible for the antioxidant properties of these plants (Mohamad et al., 2004; Shi et al., 2006; Elzaawely et al., 2007a,b). Consumption of fruits and vegetables with high contents of antioxidative phytochemicals may reduce the risk of cancer, cardiovascular disorders and many other diseases (Shui and Leong, 2006). Therefore, in the present study, additional efforts were made to evaluate the antioxidant, antigenotoxic and antimutagenic properties of EO of *A. zerumbet*.

DPPH· scavenging is a commonly used method to evaluate the ability of plant extracts to scavenge free radicals generated from the DPPH reagent (Chung et al., 2006). In this study, only non-toxic concentrations of EO were considered. The EO of *A. zerumbet* showed a significant reduction of DPPH·, starting at the lowest concentration evaluated (50 μg/mL). A high antiradical activity was observed for EO at elevated concentrations (100 and 300 μg/mL) which exhibited more than 50% reduction of DPPH· (Fig. 1). Moreover, co-treatment (EO + H_2O_2) protected leukocytes against the oxidative action of H_2O_2, as evidenced by the levels of intracellular GSH (Table 2), lipoxidation products (MDA) and ROS production, as well the low levels of oxidation of nucleotidic bases (Table 3) after treatment. Elzaawely et al. (2007a) have reported the significant presence of monoterpenes (i.e., 1,8-cineol, camphor) in the leaf oil of *A. zerumbet*. Additionally, in the present work, the chemical profiles of EO evaluated by GC–MS analysis showed monoterpenes as the predominant compounds. Among them, terpinen-4-ol (17.32%), 1,8-cineol (22.40%), γ-terpinene (11.42%) and sabinene (9.90%) were the major compounds (Table 1). There are reports that these monoterpenes show antioxidant activities (Grassmann, 2005; Juergens et al., 2009), and thus, it is likely that these compounds also contribute to the antioxidant effect of EO.

The mutagenic effects of ROS, particularly H_2O_2, which induce oxidative DNA damage, including DNA strand breaks and base modification (Valko et al., 2007), are well documented in mammalian cells (Lastra and Villegas, 2007). This study presents evidence that non-toxic concentrations of EO of *A. zerumbet* have a strong protective effect against H_2O_2-induced oxidative DNA damage in PBLs, as demonstrated by the comet and MN tests (Tables 6 and 7).

Irreparable DNA damage can activate specific mechanisms of cell death by apoptosis (Wang, 2001). The DNA lesions induced by H_2O_2 promoted apoptosis in PBLs, consequently leading to a reduction in cell viability (Figs. 4–6). Co-treatment (Fig. 4) and post-treatment (Fig. 5) with EO at concentrations ranging from 50 to 300 μg/mL increased cell viability due to reduced frequencies of apoptotic cells. Our data suggest that the antimutagenic effects during co-treatment with H_2O_2 may be due to the scavenging of free radicals and complexation of extracellular mutagenic compounds, while the protective effects in post-treatment may be due to the stimulation of DNA repair and/or reversal of DNA damage. Indeed, a number of monoterpenes, including camphor, eucalyptol and thujone, act as bioantimutagens by stimulating DNA repair (Nikolíc et al., 2011a) or act as desmutagens (i.e., linalool, myrcene and eucalyptol) through protection against oxidative DNA damage (Nikolíc et al., 2011b).

The antioxidant potential of EO correlated with its activity against the mutagenicity of H_2O_2 in PBLs, but only with treatment with EO during and after H_2O_2 exposure. Unexpectedly, in pre-treatment experiments, EO caused an increase in H_2O_2 mutagenicity or had a co-mutagenic effect (Fig. 6). In contrast, Houghton et al. (2007) reported that different extracts (aqueous and alcoholic) of *A. officinarum* and *A. galanga* induced glutathione-*S*-transferase activity in cultured hepatocytes (Hep G2 cells), and postulated that this could contribute to the antioxidant potential of *Alpinia* extracts. On the other hand, in pre-treatment protocols, washing PBLs with phosphate buffer (pH 7.4) before plating may cause a loss of nutrients and may alter the pH of the cell culture, and this could cause inactivation of antimutagenic compounds, i.e., the antioxidant components present in the EO of *A. zerumbet*.

In summary, the results obtained in this work allow us to conclude that EO of *A. zerumbet* at non-toxic concentrations (50–300 μg/mL) or dose (400 mg/kg) has no DNA damaging or mutagenic effects in cultured human leukocytes or in mice (bone marrow and whole blood) under the conditions of the assays. Also, EO from *A. zerumbet* has antioxidative and protective effect against the cytotoxicity, genotoxicity, and mutagenicity of H_2O_2. Due to the fact that *A. zerumbet* is widely used as teas and infusions, further studies designed to isolate, identify, and characterize their active antioxidant constituents should provide a greater understanding of the *in vivo* mechanisms underlying the antioxidant and antimutagenic properties of *A. zerumbet*.

Conflict of Interest

The authors declare that there are no conflict of interest.

Acknowledgements

We wish to thank CNPq, CAPES, Instituto Claude Bernard, PRONEX, FUNCAP, Banco do Nordeste and FINEP for financial support in the form of grants and fellowship awards. We are grateful to Silvana França dos Santos for technical assistance, to João Carlos C. Assunção (Universidade Federal do Ceará) for recording the GC–MS spectra and to Dr. A. Leyva for English editing of the manuscript. The authors are also grateful for scientific support and assistance from BioTechCell LTDA (Brazil).

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