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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ARTICLE INFO

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

Keywords:
Alpinia zerumbet
Antimutagenesis
Chemoprevention
Antioxidative actions

ABSTRACT

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.

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.