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Genotoxicity evaluation of kaurenoic acid, a bioactive diterpenoid present in Copaiba oil

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Abstract

Copaiba oil extracted from the Amazon traditional medicinal plant *Copaifera langsdorffii* is rich in kaurenoic acid (ent-kaur-16-en-19-oic acid), a diterpene that has been shown to exert anti-inflammatory, hypotensive, and diuretic effects in vivo and antimicrobial, smooth muscle relaxant and cytotoxic actions in vitro. This study evaluated its potential genotoxicity against Chinese hamster lung fibroblast (V79) cells in vitro, using the Comet and the micronucleus assays. Kaurenoic acid was tested at concentrations of 2.5, 5,10, 30 and 60 μg/mL. The positive control was the methylmethanesulfonate (MMS). The duration of the treatment of V79 cells with these agents was 3 h. The results showed that unlike MMS, kaurenoic acid (2.5, 5, and 10 μg/mL) failed to induce significantly elevated cell DNA damage or the micronucleus frequencies in the studied tests. However, exposure of V79 cells to higher concentrations of kaurenoic acid (30 and 60 μg/mL) caused significant increases in cell damage index and frequency. The data obtained provide support to the view that the diterpene kaurenoic acid induces genotoxicity.

Keywords: Kaurenoic acid; Plant diterpene; Comet assay; Micronucleus test; In vitro genotoxicity

1. Introduction

The medicinal plants or plants in general synthesize toxic substances, which in nature act as a defense against infections, insects and herbivores, but also may often affect the organisms that feed on them. Thus, an assessment of their cytotoxic and mutagenic potential is necessary to ensure a relatively safe use of plant-derived medicaments. Plants that belong to *Copaifera*, *Annona*, *Mikania* and *Xylopia* spp. are rich in kaurenoic acid, a diterpene that showed in vitro anti-parasitic and anti-microbial activities (Boakye-Yiadom et al., 1977; Davino et al., 1989; Batista et al., 1999; De melo et al., 2001; Wilkens et al., 2002; Cotoras et al., 2004); anti-proliferative action in tumor cell cultures and hemolytic effect against mouse and human erythrocytes (Costa-Lotufo et al., 2002; Mongelli et al., 2002); and when tested on human sperm, it reduced sperm motility, but was only weakly spermicidal (Valencia et al., 1986). Kaurenoic acid is an ent-kaurene (ent-kaur-16-en-19-oic acid) and is regarded as an intermediate in the biogenesis of the gibberellin plant growth hormone (Barton et al., 1999).

For the present investigation, kaurenoic acid (Fig. 1) was isolated from the oleoresin of *Copaifera langsdorffii* Desf. (Leguminosae) that grows abundantly in the Amazon region of Northern Brazil. The oleoresin, locally known as 'Copaiba oil' is a reputed folk remedy in its natural form for the treatment of sore throat, urinary and pulmonary infections and to hasten ulcer and wound healing (Di Stasi

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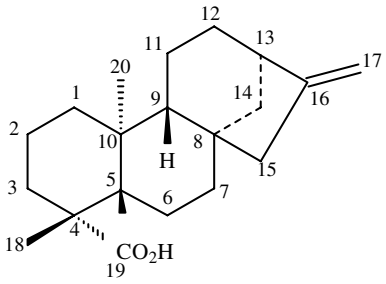

Fig. 1. Molecular structure of kaurenoic acid (ent-kaur-16-en-19-oic acid).

and Lima, 2003). It has been used as a food additive and fragrance compound and in small amounts as a flavouring agent in foods and beverages (Opdyke, 1973). Further, there have been reports on the diuretic, vasorelaxant, and anti-inflammatory effects of its major diterpene constituent kaurenoic acid in rodents (Carvalho et al., 1996; Somova et al., 2001; Paiva et al., 2003; Tirapelli et al., 2004). Despite its therapeutic potential in pre-clinical studies, so far there have been no studies on the likely genotoxic effects of this bioactive substance. Therefore, the present study evaluated the in vitro genotoxicity potential of kaurenoic acid in Chinese hamster lung fibroblasts (V79 cells) using the Comet and micronucleus assays, which are the simplified versions often followed for genotoxicity testing.

2. Materials and methods

2.1. Chemical agents

Kaurenoic acid (Fig. 1) was isolated from the oleoresin of *C. langsdorffii* Desf. (Leguminaceae) as previously described (Costa-Lotufo et al., 2002). Modified Eagle's medium (MEM), fetal calf serum, trypsin–EDTA, penicillin and streptomycin were purchased from Gibco® (Invitrogen, Carlsbad, CA, USA). Methylmethanesulfonate (MMS) and Cytochalasin-B (Cyt-B) were obtained from Sigma Aldrich Co. (St. Louis, MO, USA).

2.2. Cells and treatments

Chinese hamster lung fibroblasts (V79 cells) were cultivated under standard conditions in MEM with Earle's salts, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) (Speit et al., 1994). Cells were maintained in tissue culture flasks (Nunc, 25 cm^2) at 37 °C in a humidified atmosphere containing 5% CO_2 and were harvested by treatment with 0.15% trypsin and 0.08% EDTA in phosphate-buffered saline (PBS). Cells (3×10^5) were seeded in 5 mL of complete medium and grown for 2 days prior to treatment with the test substance. Then the medium was replaced and the substance was added as DMSO solution. Kaurenoic acid was tested at concentrations of 2.5; 5, 10, 30 and 60 μg/mL in both assays. The final concentration of DMSO in the culture medium was kept constant, below 0.1% (v/v). All cell treatments were carried out with three replicates.

2.3. Comet assay

The alkaline Comet assay was performed as described by Singh et al. (1988) with minor modifications (Hartmann and Speit, 1997; Collins, 2004). The V79 cells were incubated for 3 h with various concentrations of kaurenoic acid (2.5, 5, 10, 30, and 60 μg/mL). After this, the cells were washed with ice-cold PBS and trypsinized with 100 μL trypsin (0.15%) and were resuspended in complete medium. After this, 20 μL of cells suspension (~10^6 cells/mL) was dissolved in 0.75% low melting point agarose and immediately spread onto a glass microscope slide pre-coated with a layer of 1% normal melting point agarose. The agarose was allowed to set at 4 °C for 5 min. Then, 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, pH10.0) at 4 °C for at least 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 conducted for 20 min at 25 V (300 mA). All the above steps were conducted under yellow light or in the dark to prevent additional DNA damage. The slides were then neutralized (0.4 M Tris, pH 7.5), washed in bi-distilled water and stained using a silver staining protocol as described by Nadin et al. (2001). After staining step, the gels were dried at room temperature overnight and analyzed using an optical microscope. Hundred cells (50 cells from each of two replicate slides) were selected and analyzed for each concentration of test substance. In the selection of cells, the edges and cells around the air bubbles were avoided (Collins, 2004). These cells were scored visually according to tail length into five classes: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length 1–2x the diameter of the head; (4) class 3: with a tail longer than 2x the diameter of the head and (5) class 4: comets with no heads. A value (damage index) was assigned to each comet according to its class.

International guidelines and recommendations for the Comet assay consider that visual scoring of comets is a well-validated evaluation method. It has a high correlation with computer-based image analysis. The damage index is based on the length of migration and on the amount of DNA in the tail and is considered a sensitive measure of DNA. According to Tice et al. (2000), the damage frequency, or the proportion of cells that show tails after electrophoresis, is less informative than the damage index (DI), because it does not consider the extent of the DNA damage in the cells. Image length or migration length gives information only about the size of DNA fragments and is largely dependent upon electrophoresis conditions (i.e. voltage and duration). Thus damage index (DI) is emphasized in our analyses. The other parameters, damage frequency (DF) and image length (IL), although considered in the analysis, were used only as complementary DNA damage parameters. Damage index thus ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) (Collins et al., 1995, 1997; Silva et al., 2000). The damage frequency (%) was calculated based on number of cells with tails versus those without. The vehicle was used as negative control and MMS (4×10^{-5} M) was used as positive control.

2.4. Micronucleus test

The micronucleus assay was performed according to Matsuoka et al. (1992) with modifications (Bonacker et al., 2004). V79 cells were incubated for 3 h with various concentrations (2.5, 5, 10, 30 and 60 μg/mL) of kaurenoic acid. After treatment, the cultures were washed twice with the medium and the Cyt-B was added at final concentration of 2 μg/mL. Cultures were harvested 21 h after Cyt-B addition. The cells were separated from the bottle by trypsinization and the cellular suspension was centrifuged at 1000 rpm for 5 min. Then, cells were resuspended in a KCl 0.075 M solution maintained at 4 °C for 3 min (mild hypotonic treatment). Subsequently, the cells were centrifuged and a methanol/acetic acid (3:1) solution was gently added. This fixation step was repeated twice and finally, cells were resuspended in a small volume of methanol/acetic acid and dropped on to clean slides. The slides were stained with 10% Giemsa (pH 6.8) for 3–4 min. Slides were mounted and codified prior to the microscopic analysis. Two positive controls were used: MMS at 50 μg/mL and MNNG at 1 μg/mL. The vehicle was used as negative control. Micronuclei