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Differential effect of manool – A diterpene from *Salvia officinalis*, on genotoxicity induced by methyl methanesulfonate in V79 and HepG2 cells

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ABSTRACT

Salvia officinalis (sage) is a perennial woody subshrub native to the Mediterranean region that is commonly used as a condiment and as an anti-inflammatory, antioxidant and antimicrobial agent due to its biological activities. Manool is the most abundant micro-metabolite found in *Salvia officinalis* essential oils and extracts. We therefore decided to evaluate the cytotoxic, genotoxic and antigenotoxic potential of manool in Chinese hamster lung fibroblasts (V79) and human hepatoma cells (HepG2). Cytotoxicity was assessed by the colony-forming assay in V79 cells and toxic effects were observed at concentrations of up to 8.0 μg/mL. The micronucleus test was used to evaluate the genotoxicity and antigenotoxicity of manool in V79 and HepG2 cells at concentrations of 0.5–6.0 μg/mL and 0.5–8.0 μg/mL, respectively. For evaluation of antigenotoxicity, the concentrations of manool were combined with methyl methanesulfonate (MMS, 44 μg/mL). The results showed a significant increase in the frequency of micronuclei in cultures of both cell lines treated with the highest concentration tested, demonstrating a genotoxic effect. On the other hand, manool exhibited a protective effect against chromosome damage induced by MMS in HepG2 cells, but not in V79 cells. These data suggest that some manool metabolite may be responsible for the antigenotoxic effect observed in HepG2 cells.

1. Introduction

Salvia officinalis L. (sage) is a perennial woody subshrub native to the Mediterranean region. It is a popular species that is grown worldwide due to its culinary and medicinal uses (Farhat et al., 2009). The curative properties of sage have long been known. In Latin, *Salvia* means "to cure" and the species name, officinalis, means "medicinal" (Generalic et al., 2012).

Sage has antihydrotic, spasmolytic, antiseptic and anti-inflammatory properties and has shown beneficial effects in the treatment of mental and nervous conditions (Baricevic and Bartol, 2000). This plant has also been reported as a potential treatment for cancer (Ho et al., 2000), Alzheimer's disease, and bronchitis (Perry et al., 1999). Other biological properties of sage include antimicrobial, astringent, eupeptic and hypotensive activity (Willershausen et al., 1991), and an antimutagenic effect in *Escherichia coli* (Baricevic et al., 1996).

Diterpenes are the most characteristic metabolites of *Salvia* species and the labdane-type diterpene, manool (Fig. 1), is the major component of *S. officinalis* essential oils and extracts (Velickovic et al., 2003). Manool has recently been shown to be active against several bacteria associated with periodontitis such as *Bacteroids fragilis*, *Actinomyces naeslundii*, *Porphyromonas gingivalis*, *Peptostreptococcus anaerobius*, and *Prevotella nigrescens* (Souza et al., 2011).

In view of the relevance of natural products as a source of bioactive compounds, the ethnopharmacological use and biological activities of sage and manool are being investigated. The aim of the present study was to evaluate the cytotoxic, genotoxic and antigenotoxic potential of manool in Chinese hamster lung fibroblasts (V79 cells) and in a human hepatoma cell line (HepG2) by the cytokinesis-block micronucleus assay.

2. Material and methods

2.1. Isolation of manool

For the isolation of manool, dried leaves of certified *S. officinalis* (1.0 kg) were purchased from NutriComércio de Ervas Ltda. (São Paulo, Brazil). The species was identified by Prof. Milton Groppo and a voucher specimen (SPFR 15178) was

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deposited in the herbarium of the Department of Biology, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. The plant material was pulverized and exhaustively extracted with dichloromethane (5 L), yielding 45.5 g of the crude extract.

The extract was resuspended in 300 mL methanol/water (9:1) and filtered. The soluble fraction was partitioned with *n*-hexane (300 mL, three times), yielding 10.6 g of a hexane-soluble fraction after solvent evaporation under reduced pressure. This fraction was submitted to vacuum chromatography over silica gel 60H (500 g; Merck, art. 7736) using increasing amounts of *n*-hexane and ethyl acetate (1500 mL per fraction). The second fraction (*n*-hexane/ethyl acetate, 8:2; 2.89 g) was then partitioned by column chromatography over silica gel 60 (100 g; Merck, art. 7734) using *n*-hexane/ethyl acetate (9:1) as eluent. Fifty fractions were collected and combined after thin-layer chromatography analysis (silica gel; Whatman, art. 4420222) using *n*-hexane/ethyl acetate (9:1) as the mobile phase. The second combined fraction (200 mg) was identified as the diterpene manool.

Manool was identified by ^{1}H- and ^{13}C-NMR analysis using a Bruker DPX 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). The sample was dissolved in $CDCl_3$, and the spectra were calibrated using solvent signals at d 7.26 (^{1}H) and d 77.0 (^{13}C). The experimental values were then compared with literature data (Bastard et al., 1984; Ulubelen et al., 1997) in order to confirm the structural identification. The NMR spectra indicated a purity of manool of 95–98%.

For the subsequent experiments, manool was dissolved in dimethylsulfoxide (DMSO, 5.5 μg/mL; Sigma–Aldrich).

2.2. Cells and culture conditions

For the experiments, two different cell lines were used: normal human lung fibroblasts (V79 cells) and a human hepatoma cell line (HepG2 cells), kindly provided by the Laboratory of Mutagenesis, Department of Biological Sciences, State University of São Paulo, Araraquara, São Paulo, Brazil.

V79 cells were maintained in culture flasks (25 cm^2, Corning) containing HAM-F10 + DMEM medium (Sigma–Aldrich) and HepG2 cells in flasks containing DMEM medium (Sigma–Aldrich), both supplemented with 10% fetal bovine serum (Nutricell), 1.2 g/mL sodium bicarbonate (Sigma–Aldrich), 0.1 g/mL streptomycin (Sigma–Aldrich), and 0.06 g/mL penicillin (Sigma–Aldrich) at 37 °C in a BOD-type chamber. Under these conditions, the average cell cycle time was 12 h for V79 cells and 24 h for HepG2 cells. Both cell lines were used after the 4th passage.

2.3. DNA damage-inducing agents

The mutagen methyl methanesulfonate (MMS, 44 μg/mL; Sigma–Aldrich) was used as positive control for both V79 and HepG2 cells. MMS was previously dissolved in phosphate buffered saline (PBS), pH 7.4. Benzo[a]pyrene (B[a]P, 12.6 μg/mL; Sigma–Aldrich) was dissolved in DMSO (5.5 μg/mL) and used to evaluate the metabolic capacity of HepG2 cells. The concentrations of MMS and B[a]P were selected based on their effectiveness in inducing DNA damage (Poersch et al., 2007; Wei et al., 2010).

2.4. Colony-forming assay

The colony-forming assay in V79 cells was used to evaluate the cytotoxicity of manool. For this purpose, cell cultures were treated with concentrations of manool ranging from 1.0 to 256 μg/mL. Negative (no treatment), solvent (DMSO, 14.5 μg/mL) and positive (MMS, 110 μg/mL) controls were included. The cultures were treated for 3 h and 300 cells were seeded per culture flask (three flasks per concentration). The experiments were carried out for 10 days. At the end of the growth period, the culture medium was removed and the cells were washed with PBS, fixed in methanol/acetic acid/distilled water (1:1:8) for 30 min, and stained with 3% Giemsa for 30 min.

The colonies formed were counted with a magnifying glass to determine the survival fraction (FS, %) of cells for the different treatments (Franken et al., 2006) using the following formula:

$$FS(\%) = \frac{A}{B} \times 100$$

where A is the number of colonies found in the different treatments, and B is the number of colonies found in the negative control.

2.5. Assessment of genotoxicity and antigenotoxicity

The concentrations of manool used in the genotoxicity and antigenotoxicity studies were chosen based on the results obtained in the colony-forming assay using cytotoxicity as a selection criterion. Therefore, manool was evaluated at concentrations of 0.5, 1.0, 2.0, 4.0 and 6.0 μg/mL for V79 cells and of 0.5, 1.0, 2.0, 4.0 and 8.0 μg/mL for HepG2 cells. Different concentrations of manool (0.5, 1.0, 2.0 and 4.0 μg/mL) were combined with MMS (44 μg/mL) for antigenotoxicity assessment. Negative (no treatment), solvent (DMSO, 5.5 μg/mL) and positive (MMS, 44 μg/mL) controls were included. The protocol was performed in triplicate on three different days to ensure reproducibility.

2.6. Cytokinesis-block micronucleus assay

The cells were seeded into a culture flask containing 5 mL HAM-F10/DMEM (V79) and DMEM (HepG2) and incubated for 25 h. Next, the cells were treated with manool for 3 and 24 h, respectively, for V79 and HepG2 cells. At the end of this period, the cells were washed twice with PBS and fresh serum-supplemented medium containing 3 μg/mL cytochalasin B (Sigma–Aldrich) was added. V79 cells were incubated for an additional 17 h and HepG2 cells for 24 h.

At harvest time, the cells were rinsed with 5 mL PBS, trypsinized with 0.025% trypsin–EDTA, and centrifuged for 5 min at 900 rpm. The pellet was hypotonized in 1% sodium citrate at 37 °C and then carefully homogenized. This cell suspension was centrifuged under the same conditions. The pellet was resuspended in methanol:acetic acid (3:1) and again homogenized. Fixed cells were then transferred to slides and V79 cells were stained with 3% Giemsa and analyzed under a light microscope. HepG2 cells were stained with acridine orange (100 μg/mL) and ethidium bromide (100 μg/mL) at the time of analysis by fluorescence microscopy.

The criterion established by Fenech (2000) was used for the analysis of micronuclei. A total of 3000 binucleated cells were scored per treatment, corresponding to 1000 cells/treatment/repetition. The nuclear division index (NDI) was determined for 1500 cells analyzed per treatment, for a total of 500 cells per repetition. Cells with well-preserved cytoplasm containing 1–4 nuclei were scored. The NDI was calculated according to Eastmond and Tucker (1989) using the following formula:

$$NDI = \frac{[M1 + 2(M2) + 3(M3) + 4(M4)]}{N}$$

where $M1$–$M4$ is the number of cells with 1, 2, 3 and 4 nuclei, respectively, and N is the total number of viable cells.

Additionally, the cytotoxicity index (CI) was calculated as described by Kirsch-Volders et al. (2003):

$$CI = 100 - 100\left[\frac{NDIT - 1}{NDIC - 1}\right]$$

where $NDIT$ is the NDI found for the different treatments, and $NDIC$ is the NDI of the negative control.

2.7. Calculation of the percent reduction in DNA damage

The percent reduction in MMS-induced chromosome damage mediated by manool was calculated according to Waters et al. (1990) using the following formula:

$$\%Reduction = \frac{A - B}{A - C} \times 100$$

where A corresponds to the damage obtained for the treatment with MMS (positive control), B corresponds to the antigenotoxic treatment (manool plus MMS), and C corresponds to the negative control.

2.8. Statistical analysis

The results of the cytokinesis-block micronucleus assay were analyzed by analysis of variance, with calculation of P values. In cases in which $P < 0.05$, treatment means were compared by the Tukey test and the minimum significant difference was calculated for 0.05.

3. Results

The dose-dependent effects of manool on the viability of V79 cells were evaluated by the colony-forming assay. Fig. 2 shows the results obtained for three independent experiments. No significant differences were observed between cultures treated with 1.0, 2.0 or 4.0 μg/mL manool when compared to the negative control. However, significant differences compared to the negative control were found for manool concentrations of 8.0, 16.0, 32.0, 64.0, 128.0

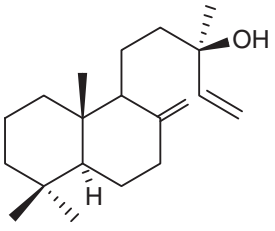

Fig. 1. Chemical structure of the diterpene manool.