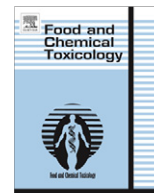
Food and Chemical Toxicology 53 (2013) 153–159

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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



Assessment of the chemopreventive effect of casearin B, a clerodane diterpene extracted from *Casearia sylvestris* (Salicaceae)

Aline M. Prieto [a], André G. dos Santos [b], Ana Paula S. Oliveira [c], Alberto J. Cavalheiro [d], Dulce H.S. Silva [d], Vanderlan S. Bolzani [d], Eliana Ap. Varanda [c], Christiane P. Soares [a,*]

[a] UNESP – Univ. Estadual Paulista, Araraquara, School of Pharmaceutical Sciences, Department of Clinical Analysis, Rua Expedicionários do Brasil 1621, Araraquara, SP, Brazil
[b] UNESP – Univ. Estadual Paulista, Araraquara, School of Pharmaceutical Sciences, Department of Natural Principles and Toxicology, Rodovia Araraquara-Jaú km 01, Araraquara, SP, Brazil
[c] UNESP – Univ. Estadual Paulista, Araraquara, School of Pharmaceutical Sciences, Department of Biological Sciences, Rodovia Araraquara-Jaú km 1, Araraquara, SP, Brazil
[d] UNESP – Univ. Estadual Paulista, Araraquara, Núcleo de Bioensaios, Biossíntese e Ecofisiologia de Produtos Naturais, Chemistry Institute, Rua Prof. Francisco Degni, s/n, Araraquara, SP, Brazil

ARTICLE INFO

Article history:
Received 12 September 2012
Accepted 17 November 2012
Available online 28 November 2012

Keywords:
Casearia sylvestris
Ames test
Comet assay
Casearin B
Antioxidant
DCFDA

ABSTRACT

Studies have shown that *Casearia sylvestris* compounds protect DNA from damage both *in vitro* and *in vivo*. Complementarily, the aim of the present study was to assess the chemopreventive effect of casearin B (CASB) against DNA damage using the Ames test, the comet assay and the DCFDA antioxidant assay. The genotoxicity was assessed by the comet assay in HepG2 cells. CASB was genotoxic at concentrations higher than 0.30 μM when incubated with the FPG (formamidopyrimidine-DNA glycosylase) enzyme. For the antigenotoxicity comet assay, CASB protected the DNA from damage caused by H_2O_2 in the HepG2 cell line in concentrations above 0.04 μM with post-treatment, and above 0.08 μM with pre-treatment. CASB was not mutagenic (Ames test) in TA 98 and TA 102. In the antimutagenicity assays, the compound was a strong inhibitor against aflatoxin B1 (AFB) in TA 98 (>88.8%), whereas it was moderate (42.7–59.4%) inhibitor against mytomicin C (MMC) in TA 102. Additionally, in the antioxidant assay using DCFDA, CASB reduced reactive oxygen species (ROS) generated by H_2O_2. In conclusion, CASB was genotoxic to HepG2 cells at high concentrations; was protective of DNA at low concentrations, as shown by the Ames test and comet assay; and was also antioxidant.

© 2012 Elsevier Ltd. Open access under the Elsevier OA license.

1. Introduction

Casearia sylvestris is a plant commonly known in Brazil as "guaçatonga," and its leaves are widely used for their anti-inflammatory and anti-ulcerative activities (Basile et al., 1990; Borges et al., 2000; Sertié et al., 2000). In addition, the bark of *C. sylvestris* has been used to treat fever symptoms and for treatment of herpes virus and diarrhea (Esteves et al., 2005). Phytochemical investigations revealed that the major compounds isolated from *C. sylvestris* (clerodane diterpenes) exhibited anti-fungal activities (Oberlies et al., 2002).

Extracts and chemical components of *C. sylvestris*, particularly clerodane diterpenes, showed cytotoxic activity against tumor cells. Specifically to the casearin B (CASB), there is only one published study concerning its biological activity, which accessed the cytotoxicity of the sample against human cell lines using MTT assay. The study presents the following CASB cytotoxicity results: MOLT-4 (leukemia; IC_{50} = 0.22 μM), MDA-MB-435 (melanoma; IC_{50} = 0.35 μM), HCT-8 (colon cancer; IC_{50} = 0.97 μM), and SF-295 (glioblastoma; IC_{50} = 0.43 μM), whereas CASB was less cytotoxic to L-929 cells (normal fibroblasts; IC_{50} = 1.52 μM) demonstrating a more selective cytotoxic response to tumor cell lines (dos Santos et al., 2010). However, there are no further mechanistic investigations concerning these results.

Regarding the effects of *C. sylvestris* compounds on DNA, studies have reported the absence of genotoxic activity of an ethanolic extract from the leaves of *C. sylvestris* in HTC (hepatoma) and V79 cells (Chinese hamster lung cells) using comet assays (Maistro et al., 2004). Additionally, the essential oil of the leaves was anticlastogenic when evaluated by observing chromosome aberrations in HTC cells (Sousa et al., 2007). Recently, a study using mouse blood cells showed that an ethanolic extract and caseargrewiin F

Abbreviations: 2-AF, 2-aminofluorene; 2-AN, 2-anthramine; AFB, aflatoxin B1; CASB, casearin B; DCFDA, dichlorodihydrofluorescein diacetate; FPG, formamidopyrimidine-DNA glycosylase; HPLC-UV, High-performance liquid chromatography-ultraviolet; IR, infrared; LPCC, low-pressure column chromatography; MMC, mytomicin C; MS, mass spectrometry; NMR, nuclear magnetic resonance; NPD, 4-nitro-o-phenylenediamine; SPE, solid-phase extraction; TLC, thin layer chromatography.

* Corresponding author. Address: UNESP, School of Pharmaceutical Sciences, Rua Expedicionários do Brasil 1621, 14 801 902 Araraquara, São Paulo, Brazil. Tel.: +55 16 33015706; fax: +55 16 33220073.
E-mail address: soarescp@hotmail.com (C.P. Soares).

0278-6915 © 2012 Elsevier Ltd. Open access under the Elsevier OA license.
http://dx.doi.org/10.1016/j.fct.2012.11.029

protected DNA from damage in low concentrations and were genotoxic and mutagenic in high concentrations (de Oliveira et al., 2009).

In fact, currently, there are a range of studies that investigate compounds isolated from plants that protect DNA from damage, elucidating the possible mechanisms by which these reductions in DNA damage occur. To complement the previous studies performed with *C. sylvestris* compounds, the aim of the present study was to assess the chemopreventive effect of CASB using the comet assay, the Ames test and an antioxidant assay.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM), antibiotic–antimycotic solution, kanamycin sulfate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (CAS # 298-93-1), dimethylsulfoxide (DMSO) (CAS # 67-68-5), formaldehyde (CAS # 50-00-0), mitomycin C (MMC) (CAS # 50-07-7), 2-aminofluorene (2-AF) (CAS # 153-78-6), 4-nitro-o-phenylenediamine (NPD) (CAS # 99-56-9), 2-anthramine (2-AN) (CAS # 613-13-8) and aflatoxin B1 (AFB) were purchased from Sigma–Aldrich® (St. Louis, Missouri, USA). Fetal calf serum (FCS) was obtained from Cultilab® (Campinas, São Paulo, BRA). Sterile H_2O_2 (CAS # 7722-84-1), ethanol (CAS # 64-17-5), silica (CAS # 7631-86-9), hexane (CAS # 110-82-7), ethyl acetate (CAS # 141-78-6) and isopropanol (CAS # 67-63-0) were purchased from Merck® (Darmstadt, Hessen, DEU). Doxorubicin (Dox) (CAS # 23214-92-8) was purchased from Eurofarma® (São Paulo, BRA). FPG endonuclease was purchased from New England Biolabs® (Ipswich, MA, USA). S9 mixture was obtained from Moltox® (Boone, NC, USA). Activated charcoal was purchased from Synth® (Diadema, São Paulo, BRA).

2.2. Preparation of extract, and purification and structure determination of CASB

Dried and powdered leaves of *C. sylvestris* (20.5 kg) were extracted with ethanol in a stainless steel extractor with solvent re-circulation for 24 h at 40 °C. The crude extract was concentrated under reduced pressure to yield 1540.0 g of dry residue. A portion (473.6 g) of the residue was separated by SPE (solid-phase extraction) from silica (60–200 μm)/activated charcoal (1:1, w/w) by elution with hexane/ ethyl acetate (95:5, v/v), ethyl acetate, and methanol to afford three fractions (SPE1-SPE3). SPE2 (16.0 g) was submitted to normal-phase LPCC (low-pressure column chromatography) over silica (40–63 μm) eluted with a gradient of hexane/ethyl acetate/ isopropanol of increasing polarity (78:20.5:1.5 to 60:37.3:2.7, v/v): 45 fractions were collected and monitored by TLC (thin layer chromatography), HPLC-UV (High-performance liquid chromatography-ultraviolet), and ^1H NMR (nuclear magnetic resonance). Fractions 15–19 were submitted to preparative reversed-phase HPLC (C18 column; 250 × 50 mm; 12 μm) with methanol/ water mixture (67:33 or 75:25, v/v) as the mobile phase to yield CASB (250.6 mg), Fig. 1. The compound was identified by spectrometric analysis using NMR, MS (mass spectrometry), IR (infrared) and UV, as described by dos Santos et al. (2010).

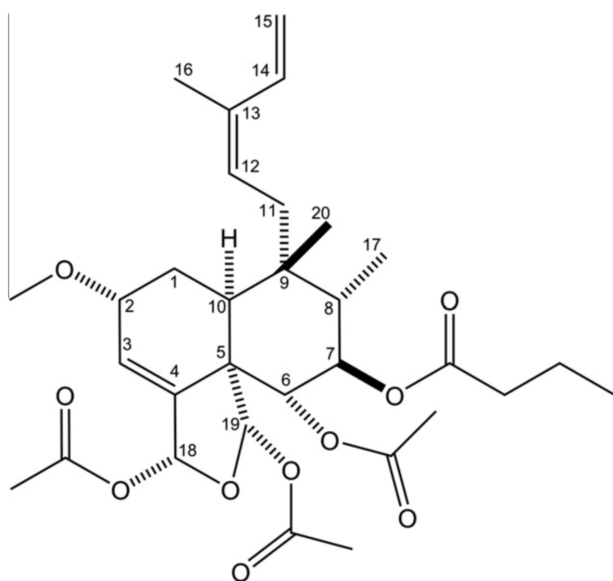


Fig. 1. Chemical structure of CASB.

2.3. Cell culture

The HepG2 cell line was purchased from the Rio de Janeiro Cell Bank, Brazil. Because this cell line was obtained from a human hepatocellular carcinoma, there are studies showing that it can express hepatic enzymes such as lipase, reductase and catalase (Busch et al., 1990; Cuthbert et al., 1997), leading to a protective effect against promutagens (Wilkening et al., 2003), making it a good model to test antioxidant properties. Additionally, HepG2 cell line showed to be highly sensitive toward several genotoxicants that give negative results in other *in vitro* assays such as mycotoxins (Knasmüller et al., 2004a), becoming a very suitable tool for genotoxicity testing (Knasmüller et al., 2004b). Additionally, there are no studies concerning if CASB is a phase I/II inductor, which makes HepG2 more suitable to test CASB since there are studies that show that HepG2 cell line is sensitive to both phase I and II inductors genotoxicants (Majer et al., 2004). The cells were grown in 75 cm^3 flasks (Techno Plastic Products®, Trasadingen, CHE) with loosened caps, containing DMEM supplemented with 10% FCS (v/v), antibiotic–antimycotic Solution (1000 U of penicillin, 100 μg/mL of streptomycin sulfate and 0.25 μg/mL amphotericin B), and kanamycin sulfate (100 μg/mL) at 37 °C in a CO_2 incubator (Thermo Fisher Scientific Inc.®, MA, USA) under a humidified atmosphere composed of 5% CO_2 in 95% air.

2.4. Viability

As proposed by Tice et al. (2000), the comet assay must be performed in conditions of low cytotoxicity; consequently the genotoxicity and antigenotoxicity of the HepG2 cells were assessed under the IC_{20} obtained from the MTT assay. The antimutagenicity was assessed under non-cytotoxic conditions using the Ames test, as previously performed in *Salmonella typhimurium* strains.

2.4.1. MTT assay

For the MTT assay, 10^4 cells/well were seeded in a 96 well plate, then the cells were exposed to at least five different concentrations of CASB compounds for a period of 24 h in triplicate, for three independent experiments. The treatments were: (1) negative control (DMEM), (2) positive control (Dox at 15 μg/mL), (3) vehicle control (DMEM plus DMSO 0.05% (v/v)), (4) five different concentrations of CASB (0.65–10.00 μM). After the treatments, the MTT assay was performed as described by Mosmann (1983). Finally, the viability was obtained using the following formula: viability (%) = (treatments × 100)/(negative control).

2.4.2. Viability in

S. typhimurium First, the culture medium, bacteria (TA98 or TA102), sterile physiological solution and the compound to be tested were homogenized by vortexing. Subsequently, the mixture was added to tubes with 9 mL of sterile physiological solution to obtain a dilution of 1:10, which was homogenized, and a final volume of 1 mL was transferred to another tube with the same amount of physiological solution; this procedure was repeated to obtain a dilution of 10^{-5}. From the tube containing a dilution of 10^{-5}, 0.1 mL was seeded on nutrient agar plates, then incubated for 24 h at 37 °C. After this period, the colonies were counted and the colony-forming units/mL was calculated (CFU = colonies × dilution × 10). The percentage of viability for each treatment was compared to the number of colonies from the negative control. According to Vargas et al. (1993), cytotoxicity was considered for samples with a percentage of viability below 60%.

2.5. Comet assay

2.5.1. Genotoxicity

HepG2 cells were seeded in 24-well plates (5 × 10^4 cells/well) and treated for 24 h with five concentrations of CASB using 1:2 dilution ratio. The treatments for the comet assay were performed to allow the cells to complete at least one entire cell cycle (24 h); in this way, it was possible to assess the damages that the cells were not able to repair. The CASB concentrations tested were: 0.15, 0.30, 0.60, 1.20 and 2.30 μM. DMEM plus 0.05% DMSO and 0.5% Hank's solution (v:v) was used as vehicle control (VC); for the negative control (NC), the cells were treated only with DMEM; and for the positive control, the cells were treated with 1 mM H_2O_2 for 10 min. The concentration of hydrogen peroxide was obtained by absorbance as proposed by Brestel (1985). After the treatments, the cells were detached, re-suspended and homogenized with low-melting-point agarose, spread on a microscope slide pre-coated with normal-melting-point agarose and covered with a coverslip. Then, the slides were incubated for 30 min with 90 μL of FPG enzyme (1:3000, v:v) at 37 °C, to specifically assess the oxidative damage in the DNA (Collins et al., 2008). Additionally, an experiment without FPG incubation was also performed. The alkaline version of the comet assay (single cell gel electrophoresis) was performed as described by Singh et al. (1988). Duplicate slides were prepared and stained with ethidium bromide, and 50 cells were screened per sample with a fluorescent microscope (ZEISS®, Jena, Thuringia, DEU) equipped with an excitation filter of 515–560 nm, a barrier filter of 590 nm and a 40× objective. The level of

DNA damage was assessed by an image analysis system (TriTek CometScore® 1.5, 2006, Sumerduck, VA, USA), and the DNA percent in the tail was obtained for each treatment. The groups treated with the compounds were compared with the vehicle control using the Kruskal–Wallis test and the associated Dunn post-test using the OriginPro 8.0 software (OriginLab®, Northampton, MA, USA).

2.5.2. Antigenotoxicity

HepG2 cells were pre-treated with CASB and were then exposed to 1 mM of H_2O_2 for 10 min, or the cells were exposed to H_2O_2 1 mM for 10 min and post-treated with the CASB. Five concentrations were tested: 0.02, 0.04, 0.08, 0.15 and 0.30 μM. The positive control consisted of treating with H_2O_2 (1 mM) for 10 min, and the cells were pre- or post-treated with DMEM, negative control (DMEM) and vehicle control (cells pre- or post-treated with 0.05% DMSO plus Hank's 0.5% in DMEM plus H_2O_2 (1 mM)). Subsequently, the comet assay was performed as previously described for the genotoxicity assay, incubating the slides with FPG enzyme (1:3000, v:v) because the mutagen used in this test was H_2O_2. Statistical analysis was performed using the Kruskal–Wallis test and the associated Dunn post-test to compare the treated groups with the vehicle control group using the OriginPro 8.0 software (OriginLab®, Northampton, MA, USA). The damage inhibition percent (I) was calculated as proposed by Hosseinimehr and Karami (2005): I = [(Positive control) − (Test)] × 100/(Positive control). Additionally, the percentage of inhibition was classified as strong (higher than 60%), moderate (60–41%), weak (40–21%) and negligible (20–0%) (Calomme et al., 1996).

2.6. Ames test

2.6.1. Mutagenicity

Mutagenicity was evaluated by the *Salmonella*/microsome assay that is based on the plate-incorporation method proposed by Maron and Ames (1983), using *S. typhimurium* test strains TA98 and TA102, provided by Dr. B.N. Ames (Berkeley, CA, USA), both with and without metabolization by the S9 mixture. The test strains were obtained from frozen cultures and were grown overnight for 12–14 h at 37 °C in oxoid nutrient broth No. 2. The CASB was dissolved in DMSO and added to 2 mL of top agar mixed with 100 μL of bacterial culture (1–2 × 10^8 cells/mL) and were then added to a plate with minimal agar. Then, the plates were incubated at 37 °C for 48 h and the number of His+ revertant colonies was manually counted. Additionally, the influence of metabolic activation was assessed by adding 500 μL of S9 mixture (S9 at a concentration of 0.04 mL/mL of mix). All experiments were analyzed in triplicate. The standard mutagens used as positive controls in each experiment were: TA 102 (−S9) MMC (0.5 μg/plate), TA 102 (+S9) 2-AF (10 μg/plate), TA 98 (−S9) NPD (10 μg/plate), TA98 (+S9) 2-AN (1.25 μg/plate). DMSO was used as the negative (vehicle) control (100 μL – maximum volume that was used in the assay). We selected the concentrations based on the toxicity. In a preliminary test, we considered toxicity to be when we observed either a reduction in the number of his+ revertants, or an alteration in the auxotrophic background (i.e., background lawn). In all subsequent assays, the upper limit of the dose range tested was the highest nontoxic dose or the lowest toxic dose determined in this assay. In the mutagenicity assay, we used the following concentrations: 0.03; 0.05; 0.10, 0.15 and 0.21 mg/plate. Statistical analysis was performed using the Salanal software (Integrate Laboratory Service, NC, USA, 1992), adopting the Bernstein et al. (1982) model. The mutagenic index (MI) was also calculated for each dose; that is, the average number of revertants per plate divided by the average number of revertants per plate in the negative (solvent) control. A sample was considered positive when MI $\geqslant$ 2 for at least one of the tested doses, and there was a reproducible dose–response curve (Varella et al., 2004).

2.6.2. Antimutagenicity

Before assessing the antimutagenicity, the cytotoxicity of the highest dose of the compounds associated with the mutagen was tested. Based on the results of this preliminary assay, the *C. sylvestris* components were tested in non-cytotoxic doses for their antimutagenic properties. The procedures for the antimutagenicity assays were similar to those described for the mutagenicity assays, except that in each tube of top agar containing the bacterial strain and the CASB, the mutagenic agent was also added. One of the mutagens tested was a direct-acting genotoxic compound, MMC in TA 102 (4 × 10^{-7} mg/plate); while the other was the indirect-acting promutagen AFB in TA 98 (5 × 10^{-7} mg/plate). In addition to AFB, the promutagen-activating fraction S9 was added. This fraction was prepared and used following Maron and Ames (1983). Toxicity to the various bacteria was also tested, and the sample concentrations employed for the antimutagenic test were all found to be non-toxic. The following CASB concentrations were used in the antimutagenicity assay: 0.02; 0.03; 0.05; 0.10 mg/plate. The concentration of CASB that inhibits 50% of mutagenicity (MID_{50}) was obtained using regression analysis (OriginPro 8.0 software – OriginLab®, Northampton, MA, USA). The calculation of the percentage of mutagenic inhibition (I) was carried out as described by Tachino et al. (1994). We also classified the mutagenic inhibition as described in antigenotoxicity assay.

$$I = [1 - (\text{revertants per plate with inhibitor}/\text{revertants per plate without inhibitor})] \times 100$$

2.7. Antioxidant assay

The intracellular reactive oxygen species (ROS) were measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). Inside the cell, DCFDA (inactive ROS) is converted to DCFH (active ROS) by an esterase. H_2O_2 and other ROS oxidize intracellular DCFH (non-fluorescent) to DCF (fluorescent). HepG2 cells (10^4 cells/well) were seeded in sterile black plates. Then, the cells were treated with CASB in three concentrations: 0.04, 0.08 and 0.15 mM for 24 h with their respective controls. The concentrations of this compound were chosen based on previously obtained results of cytotoxicity and genotoxicity assays. Additionally, the following controls were used for the experiment: (1) negative control (NC) cells (cells maintained in culture medium), (2) vehicle control (VC) (cells treated with 0.05% DMSO plus Hank's 0.5% in DMEM) and (3) quercetin (a natural antioxidant) control (Q) (40 mM). After treatment, the wells were washed twice with Hank's solution. Next, 100 μL of a solution of 5 mM DCFDA diluted in Hank's was added and incubated for 30 min at 37 °C while protected from light. Thereafter, 1 mM hydrogen peroxide was added for 10 min, after which time the hydrogen peroxide was removed from the plate by aspiration. The wells were washed twice with Hank's, and 100 μL of Hank's solution was added to each well for reading with a fluorimeter. Reading was performed every minute for 30 min, at excitation: λ = 485 nm, emission: λ = 528 nm. A curve of fluorescence intensity *versus* time was plotted, and from this curve, the total fluorescence intensity for each treatment was estimated by obtaining the integral of each curve (area under the curve) using the OriginPro 8.0 software (OriginLab®, Northampton, MA, USA). Three independent experiments were performed in triplicate. Next, a one-way analysis of variance (ANOVA) was performed with the Dunnett post-test to compare the total intensities obtained from the treatments *versus* the vehicle control (VC) (Nakajima et al., 2009).

3. Results

3.1. Viability and genotoxicity

Before the effect of the compounds on the DNA in the HepG2 cell line was assessed, the MTT assay was performed. The assays were conducted under the $IC_{20,}$ 2.3 μM (Fig. 2). Complementarily, the results, shown in Fig. 2, demonstrate that this compound was genotoxic to HepG2 cells at concentrations above 0.30 μM when incubated with FPG enzyme, whereas without the enzyme incubation, CASB begins to show genotoxicity at 1.15 μM (Fig. 3).

3.2. Antigenotoxicity

To assess antigenotoxicity, concentrations that slightly affected the cell viability and that were not genotoxic were chosen. CASB significantly reduced the DNA damage caused by H_2O_2 in HepG2 cells in both pre- (0.08 and 0.15 μM) and post-treatments (0.04, 0.08 and 0.15 μM) (Fig. 4A). With regard to the percent inhibition of DNA damage (Fig. 4B), the post-treatment was the most

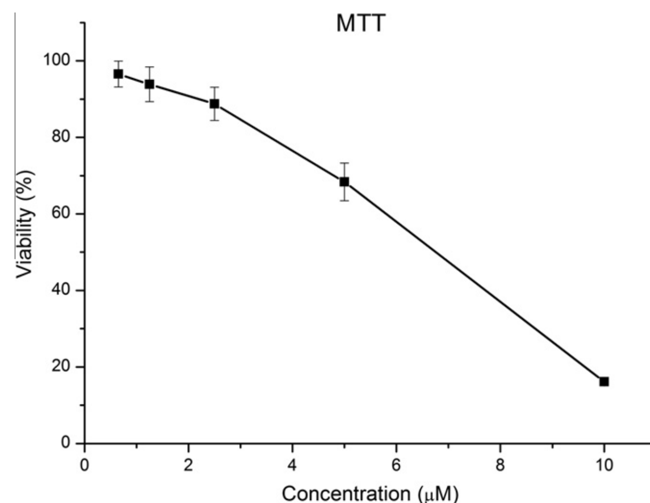


Fig. 2. MTT viability assay of CASB in the HepG2 cell line treated with the compound for 24 h. IC_{20} = 1.15 μM.

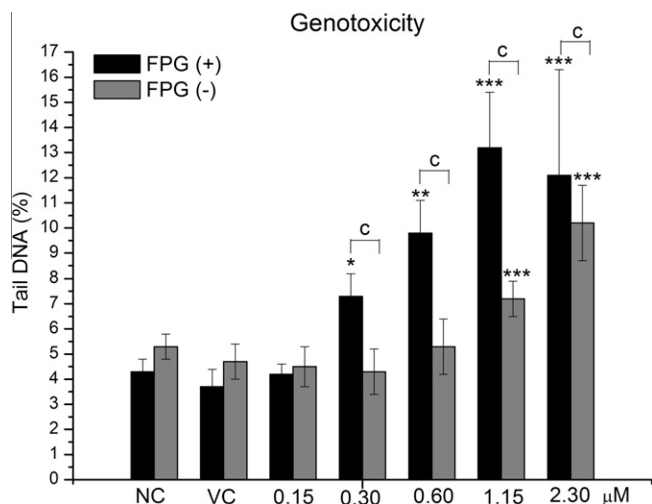


Fig. 3. Genotoxicity in the HepG2 cell line exposed to CASB for 24 h assessed by the comet assay, with and without incubation with FPG enzyme. NC = negative control (cells in cell culture medium, DMEM); VC = vehicle control (cells treated with DMSO 0.05%, Hank's 0, 5% in DMEM). Kruskal–Wallis with Dunn post-test VC *versus* treatments: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Mann–Whitney FPG(+) *versus* FPG(−): $^{c}p < 0.001$.

efficient, presenting strong inhibition (70.1%) in the higher concentration tested (0.15 μM). At the same concentration, the pre-treatment exhibited moderate inhibition (47.8%).

3.3. Mutagenicity

Table 1 shows that CASB was not mutagenic to *S. typhimurium* strains TA 98 and TA 102 with and without metabolization. That the mutagenic index was <2 demonstrates that the compound tested could not induce an increase in the number of revertants.

3.4. Antimutagenicity

The results of the antimutagenicity assay for CASB are shown in Table 2. The compound was used in association with MMC and AFB in the strain TA 98 under metabolization (+S9) and in the strain TA 102 without metabolization (−S9). The combination of CASB (0.10 mg/plate) with the mutagens AFB and MMC resulted in a viability of 100% and 69%, respectively, which are acceptable to perform the antimutagenicity assay. Under metabolization using the strain TA 98, CASB was a strong inhibitor (approximately 90%) against DNA damage induced by AFB. For the strain TA 102 without metabolization, CASB was a weak (40–21%) or moderate (41–60%) inhibitor against the mutagenicity induced by MMC.

3.5. Antioxidant assay

The results of the antioxidant assay (Fig. 5) show that CASB was able to significantly reduce ROS generated by H_2O_2 at the two higher concentrations tested (0.08 and 0.15 μM) compared with the vehicle control (Fig. 5B). These results corroborate the H_2O_2 DNA damage reduction shown in the post-treatment with CASB assessed by the comet assay, as shown in Fig. 4A.

4. Discussion

The present study assessed the mutagenic/genotoxic, antimutagenic/antigenotoxic and antioxidant activities of CASB in *S. typhimurium* and HepG2 cell lines. Recently, natural products have been suggested to possess DNA protective and antioxidant properties (Dellai et al., 2009; Gupta et al., 2009), which can be useful to prevent diseases such as cancer.

The *S. typhimurium* strains TA 98 and TA 102 were used in the Ames test. The TA 98 strain has a specific deletion in the plasmid pKM 101 that makes this strain reversible in frameshift events (Maron and Ames, 1983). *S. typhimurium* TA 102 is susceptible to oxidative damage due to a mutation in the gene *G428* (Levin et al., 1982).

CASB was not mutagenic to the strains TA 98 and TA 102. As described previously, these results indicate that the compound did not induce significant frameshift events or oxidative damage at the concentrations tested.

However, CASB was genotoxic at concentrations below the IC_{20} to the HepG2 cells. Interestingly, when the enzyme FPG was used, the genotoxicity increased significantly compared to the assay

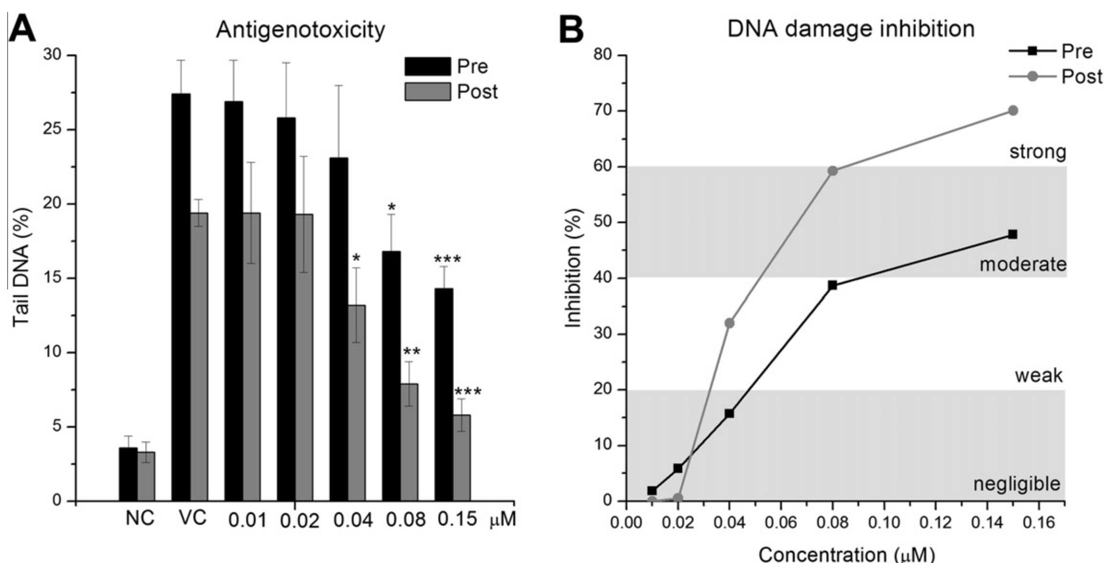


Fig. 4. (A) Antigenotoxicity in the HepG2 cell line both pre- and post-treated with CASB for 24 h against the damage caused by H_2O_2 (1 mM), which was assessed by the comet assay and incubated with FPG enzyme. NC = negative control (cells in cell culture medium, DMEM); VC = vehicle control (cells pre- or post-treated with DMSO 0.05%, Hank's 0, 5% in DMEM and exposed to H_2O_2). Kruskal–Wallis with Dunn post-test VC *versus* treatments: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (B) Inhibition of H_2O_2 DNA damage by the antigenotoxicity treatment described in A. Negligible (20–0%), weak (40–21%), moderate (60–41%) and strong (higher than 60%).

Table 1
Mutagenic activity of CASB in strains of S. typhimurium TA 98 and TA 102 in the presence (+S9) and absence (−S9) of metabolization.

mg/plate	*Salmonella typhimurium* revertants/plate ±SD (MI)			
	TA 98		TA 102	
	−S9	+S9	−S9	+S9
DMSO	71.0 ± 5.6	70.3 ± 11.2	208.0 ± 14.4	317.3 ± 20.2
0.03	78.3 ± 10.3 (1.1)	76.0 ± 16.2(1.1)	211.3 ± 54.3 (1.0)	284.3 ± 3.1 (0.9)
0.05	74.7 ± 15.1 (1.1)	94.3 ± 19.9 (1.3)	217.0 ± 23.5 (1.0)	296.0 ± 24.3 (0.9)
0.10	76.0 ± 12.1 (1.1)	79.0 ± 24.4 (1.1)	188.3 ± 34.1 (0.9)	273.7 ± 13.8 (0.9)
0.15	77.7 ± 20.5 (1.1)	94.3 ± 9.3(1.3)	179.0 ± 24.6 (0.9)	297.7 ± 35.3 (0.9)
0.21	59.3 ± 7.4 (0.8)	88.7 ± 2.1 (1.3)	193.3 ± 33.7 (0.9)	260.7 ± 41.4 (0.8)
Control +	1749.3 ± 152.5	460.0 ± 20.0	1438.3 ± 160.3	371 ± 35.7

MI = Mutagenesis index; SD = standard deviation; Control +: TA 102 (−S9) MMC (0.5 μg/plate), TA 102 (+S9) 2-AF (10 μg/plate), TA 98 (−S9) NPD (10 μg/plate), TA98 (+S9) 2-AN (1.25 μg/plate). DMSO (100 μL/plate) = negative control.

Table 2
Antimutagenicity assay. *S. typhimurium* revertants per plate treated with CASB in association with mitomycin C (MMC) and aflatoxin B1 (AFB) in strain TA 98 under metabolization (+S9) and strain TA 102 without metabolization (−S9).

Salmonella typhimurium revertants/plate ±SD (I)					
TA 98 (+S9) mg/plate			TA 102 (−S9) mg/plate		
AFB (5×10^{-7})	349.7 ± 0.6		MMC (4×10^{-7})	690.7 ± 90.9	
DMSO	30.3 ± 3.1		DMSO	209.3 ± 10.1	
CASB + AFB (5×10^{-7})		MID_{50}	CASB + MMC (4×10^{-7})		MID_{50}
0.02	34.3 ± 4.5 (90.2)[d]	0.003	0.02	650.7 ± 101.9 (5.8)[a]	0.04
0.03	39 ± 6 (88.8)[d]		0.03	364 ± 31.4 (47.3)[c]	
0.05	33.3 ± 2.5 (90.5)[d]		0.05	395.7 ± 47.2 (42.7)[c]	
0.1	35.7 ± 1.5 (89.8)[d]		0.1	280.7 ± 4.2 (59.4)[c]	

I = percent inhibition; SD = standard deviation; CASB = CASB; DMSO (100 μL/plate) = negative control; I = [a]negligible (20–0%), [b]weak (40–21%), [c]moderate (60–41%) and [d]strong (higher than 60%); MID_{50} = concentration of CASB (mg\plate) that inhibits 50% of mutagenicity.

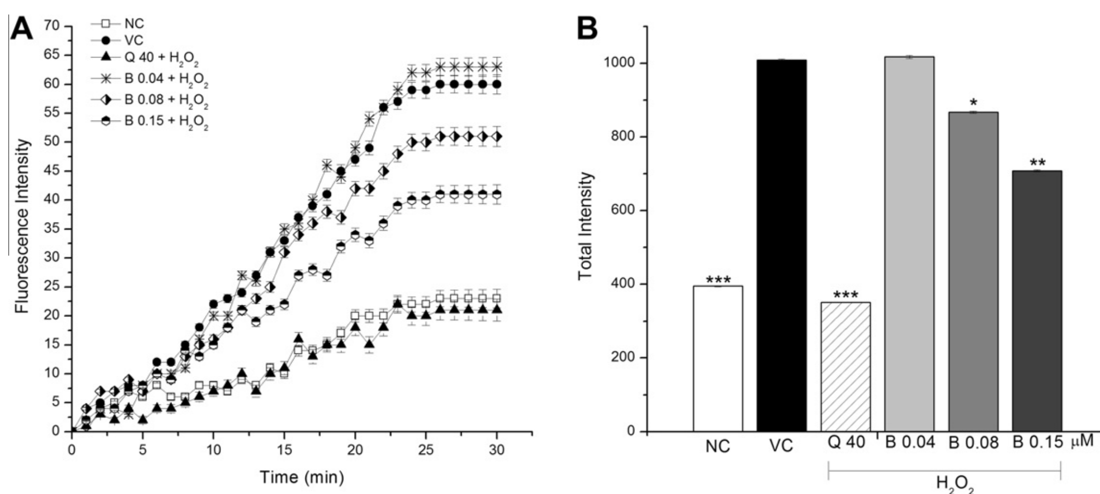


Fig. 5. Antioxidant activity using DCFDA in the HepG2 cell line pre-treated for 24 h with CASB against ROS generated by H_2O_2. (A) Curve of fluorescence intensity obtained in 30 min of reaction. (B) Total intensity obtained by the integral of the curves presented in A. NC = negative control (cells in cell culture medium, DMEM); VC = vehicle control (cells pre-treated with DMSO 0.05%, Hank's 0.5% in DMEM and exposed to H_2O_2). Q = quercetin control (40 μM). B 0.04 = CASB 0.04 μM. B 0.08 = CASB 0.08 μM. B 0.15 = CASB 0.15 μM. Kruskal–Wallis with Dunn post-test VC *versus* treatments: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

without enzyme incubation (Fig. 3). Thus, CASB itself caused oxidative damage to DNA with a concentration–response profile. Although this mechanism has not been elucidated for CASB, de Carvalho et al. (1998) demonstrated that two different diterpene clerodanes extracted from *C. sylvestris*, known as casearins S and T, caused acetylation of the DNA molecule when measured by DNA damage detection in cells of *Saccharomyces cerevisiae* mutants. Therefore, the interactions of casearins with DNA may be associated with the genotoxic effects observed in this study.

Additionally, Zeiger (2007) notes that DNA damage protection studies have to be conducted in parallel with mutagenicity and cytotoxicity assays to guarantee their safety and avoid overestimation of the chemopreventive effects observed, respectively. Thus, our DNA damage protection studies were performed using non-cytotoxic and non-genotoxic/mutagenic conditions.

Compounds that protect DNA from damage can act by two different mechanisms. They can be considered desmutagens, which reduce the mutagenic/genotoxic effect by directly interacting with

the mutagens, block their effects by inhibiting their metabolic activation or enhance their detoxification. The pre-treatment is commonly performed to assess the detoxificant properties of the compounds, since detoxificant enzymes can be induced during the pre-treatment period. Alternatively, these compounds can be classified as bioantimutagens, which promote DNA repair after damage, increase DNA replication, inhibit error-prone replication or suppress the growth and replication of cells with damaged DNA .The post-treatment is useful to assess bioantimutagenic effects, for instance DNA repair response can be activated after damage, and the post-treatment can help to improve this effect (Kada and Shimoi, 1987). The results obtained for CASB are interesting because in the antigenotoxicity assay in the HepG2 cell line, CASB protected the DNA against oxidative damage at lower concentrations with both pre- and post-treatment (Fig. 4). Additionally, the compound also protected the DNA against oxidative damage in TA102 when exposed to MMC (Table 2).

Taken together, the data shown in the genotoxicity and antigenotoxicity assays are particularly interesting. They corroborate previous results obtained in animal models for compounds from *C. sylvestris*, where a weak genotoxic response and an antigenotoxic activity were observed concurrently in the comet assay at the same concentrations (de Oliveira et al., 2009). In addition, other studies described the same profile for compounds derived from natural products (Miyaji et al., 2004). Specifically, with regard to derivatives of *C. sylvestris*, the possible acetylation of DNA that generates a genotoxic response may also be responsible for the acetylation of histones. It is already known that histone acetylation results in DNA relaxation; thus, the DNA becomes accessible to the transcriptional machinery (Sterner and Berger, 2000), thereby enabling the correction of lesions, either by the DNA repair mechanism or by the transcription of detoxificant enzymes (Li et al., 2007). Concerning the detoxificant activity, the results of post-treatment with CASB in the comet assay (Fig. 4) can be explained by the antioxidant DCFDA assay results, which show that CASB significantly induced antioxidant enzymes that reduce ROS generated by H_2O_2 (Fig. 5). This finding explains why both a desmutagenic and a bioantimutagenic response were observed in this work, and why de Oliveira et al. (2009) observed protection to damage induced by cyclophosphamide in an animal model.

Complementarily, to the comet assay the use of 24 h treatment was performed to allow the cell to complete one entire cell cycle, consequently, it was possible to express DNA repair machinery (post-treatment) or induce antioxidant enzymes (pre-treatment). Because of this, there was a difference in the percent DNA damage in the comet tail observed in both pre- and post-treatments that was clearly observed in the vehicle controls. The explanation for this result is that the pre-treatment consists of treating cells with vehicle control or compound, then inducing genotoxic damage followed by the comet assay. Damage occurs, but the repair system of the cell does not have enough time to repair it; therefore, higher values of damage are observed. However, with the post-treatment, damage is generated and then the treatments are added for 24 h, thus allowing the time required for the cell to repair any damage spontaneously, resulting in the vehicle control showing less damage after the post-treatment compared to pre-treatment. However, there is significant residual damage (shown in vehicle control) that the cell cannot repair itself, and the possible reduction of this damage is evaluated with the post-treatment of cells with the compounds.

Additionally, compared to other compounds that are antimutagenic and antioxidant, such as *N*-acetyl-L-cysteine (MID_{50} = 0.07 -mg/plate; TA 100; against 4-nitroquinoline 1-oxide) or myrcetin, a flavonoid extracted from natural products (MID_{50} = 0.16 mg/plate; TA 98; S9 incubation, against cigarette smoke condensate) (Camoirano et al., 1994), CASB was most efficient on protecting DNA from damage once it presented a MID_{50} lower than *N*-acetyl-L-cysteine or myrcetin as shown in Table 2: (MID_{50} = 0.04 mg/plate; TA 102; against MMC); (MID_{50} = 0.003 mg/plate; TA 98; S9 incubation; against AFB).

In the Ames test, CASB strongly inhibited the effects of AFB in the TA 98 strain under metabolization in the antimutagenesis assay. These results indicate that CASB interacts with AFB and may inhibit the microsomal activation of AFB to electrophilic metabolites (Ammar et al., 2008). In the TA 102 strain, a lower inhibition of the direct agent MMC was observed when compared to AFB. This result demonstrates that CASB is highly efficient in protecting DNA from damage under a metabolization model.

In conclusion, CASB showed interesting chemopreventive characteristics in both HepG2 cells and *S. typhimurium* strain by protecting DNA against different types of damage and by acting as an antioxidant through inducing detoxificant enzymes in HepG2 cells.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by grants from the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP, São Paulo Research Foundation; Grant no. 2008/58908-0 to A.M.P and 2009/52481-7 to C.P.S), from the Biota-FAPESP Program (Grant no. 2003/02176-7 to V.S.B.), from the BIOprospecTA Program (Grant no. 2004/07932-7 to D.H.S.S. and A.J.C.), and from the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq, National Council for Scientific and Technological Development; Grant no. 305615/2006-8 to C.P.S.; scholarship grant to A.G.S.). We wish to thank Silvia Helena de Oliveira David for her excellent technical assistance.

References

Ammar, R.B., Sghaier, M.B., Boubaker, J., Bhouri, W., Naffeti, A., Skandrani, I., Bouhlel, I., Kilani, S., Ghedira, K., Chekir-Ghedira, L., 2008. Antioxidant activity and inhibition of aflatoxin B1-, nifuroxazide-, and sodium azide-induced mutagenicity by extracts from *Rhamnus alaternus* L.. Chem. Biol. Interact. 174, 1–10.

Basile, A.C., Sertie, J.A., Panizza, S., Oshiro, T.T., Azzolini, C.A., 1990. Pharmacological assay of *Casearia sylvestris*. I: Preventive anti-ulcer activity and toxicity of the leaf crude extract. J. Ethnopharmacol. 30, 185–197.

Bernstein, L., Kaldor, J., McCann, J., Pike, M.C., 1982. An empirical approach to the statistical analysis of mutagenesis data from the *Salmonella* test. Mutat. Res. 97, 267–281.

Borges, M.H., Soares, A.M., Rodrigues, V.M., Andriao-Escarso, S.H., Diniz, H., Hamaguchi, A., Quintero, A., Lizano, S., Gutierrez, J.M., Giglio, J.R., Homsi-Brandeburgo, M.I., 2000. Effects of aqueous extract of *Casearia sylvestris* (Flacourtiaceae) on actions of snake and bee venoms and on activity of phospholipases A2. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 127, 21–30.

Brestel, E.P., 1985. Co-oxidation of luminol by hypochlorite and hydrogen peroxide implications for neutrophil chemiluminescence. Biochem. Biophys. Res. Commun. 126, 482–488.

Busch, S.J., Barnhart, R.L., Martin, G.A., Flanagan, M.A., Jackson, R.L., 1990. Differential regulation of hepatic triglyceride lipase and 3-hydroxy-3-methylglutaryl-CoA reductase gene expression in a human hepatoma cell line, HepG2. J. Biol. Chem. 265, 22474–22479.

Calomme, M., Pieters, L., Vlietinck, A., Vanden Berghe, D., 1996. Inhibition of bacterial mutagenesis by Citrus flavonoids. Planta Med. 62, 222–226.

Camoirano, A., Balansky, R.M., Bennicelli, C., Izzotti, A., D'Agostini, F., 1994. Experimental databases on inhibition of the bacterial mutagenicity of 4-nitroquinoline 1-oxide and cigarette smoke. Mutat. Res. 317, 89–109.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Stetina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143–151.

Cuthbert, C., Wang, Z., Zhang, X., Tam, S.P., 1997. Regulation of human apolipoprotein A-I gene expression by gramoxone. J. Biol. Chem. 272, 14954–14960.

de Carvalho, P.R., Furlan, M., Young, M.C., Kingston, D.G., Bolzani, V.S., 1998. Acetylated DNA-damaging clerodane diterpenes from *Casearia sylvestris*. Phytochemistry 49, 1659–1662.

de Oliveira, A.M., dos Santos, A.G., Dos Santos, R.A., Csipak, A.R., Olivato, C., da Silva, I.C., de Freitas, M.B., Bassi, C.L., Cavalheiro, A.J., Bolzani, V.S., Silva, D.H., Sakamoto-Hojo, E.T., Takahashi, C.S., Soares, C.P., 2009. Ethanolic extract of *Casearia sylvestris* and its clerodane diterpen (caseargrewiin F) protect against DNA damage at low concentrations and cause DNA damage at high concentrations in mice's blood cells. Mutagenesis 24, 501–506.

Dellai, A., Mansour, H.B., Limem, I., Bouhlel, I., Sghaier, M.B., Boubaker, J., Ghedira, K., Chekir-Ghedira, L., 2009. Screening of antimutagenicity via antioxidant activity in different extracts from the flowers of *Phlomis crinita* Cav. ssp. *mauritanica* munby from the center of Tunisia. Drug Chem. Toxicol. 32, 283–292.

dos Santos, A.G., Ferreira, P.M., Vieira Junior, G.M., Perez, C.C., Gomes Tininis, A., Silva, G.H., Bolzani Vda, S., Costa-Lotufo, L.V., Pessoa Cdo, O., Cavalheiro, A.J., 2010. Casearin X, its degradation product and other clerodane diterpenes from leaves of *Casearia sylvestris*: evaluation of cytotoxicity against normal and tumor human cells. Chem. Biodivers. 7, 205–215.

Esteves, I., Souza, I.R., Rodrigues, M., Cardoso, L.G., Santos, L.S., Sertie, J.A., Perazzo, F.F., Lima, L.M., Schneedorf, J.M., Bastos, J.K., Carvalho, J.C., 2005. Gastric antiulcer and anti-inflammatory activities of the essential oil from *Casearia sylvestris* Sw. J. Ethnopharmacol. 101, 191–196.

Gupta, D.K., Nicoloso, F.T., Schetinger, M.R., Rossato, L.V., Pereira, L.B., Castro, G.Y., Srivastava, S., Tripathi, R.D., 2009. Antioxidant defense mechanism in hydroponically grown Zea mays seedlings under moderate lead stress. J. Hazard. Mater. 172, 479–484.

Hosseinimehr, S.J., Karami, M., 2005. Citrus extract modulates genotoxicity induced by cyclophosphamide in mice bone marrow cells. J. Pharm. Pharmacol. 57, 505–509.

Kada, T., Shimoi, K., 1987. Desmutagens and bio-antimutagens – their modes of action. BioEssays 7, 113–116.

Knasmüller, S., Cavin, C., Chakraborty, A., Darroudi, F., Majer, B.J., Huber, W.W., Ehrlich, V.A., 2004a. Structurally related mycotoxins ochratoxin A, ochratoxin B, and citrinin differ in their genotoxic activities and in their mode of action in human-derived liver (HepG2) cells: implications for risk assessment. Nutr. Cancer 50, 190–197.

Knasmüller, S., Mersch-Sundermann, V., Kevekordes, S., Darroudi, F., Huber, W.W., Hoelzl, C., Bichler, J., Majer, B.J., 2004b. Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. Toxicology 198, 315–328.

Levin, D.E., Hollstein, M., Christman, M.F., Schwiers, E.A., Ames, B.N., 1982. A new *Salmonella* tester strain (TA102) with A $\times$ T base pairs at the site of mutation detects oxidative mutagens. Proc. Natl. Acad. Sci. U.S.A 79, 7445–7449.

Li, B., Carey, M., Workman, J.L., 2007. The role of chromatin during transcription. Cell 128, 707–719.

Maistro, E.L., Carvalho, J.C., Mantovani, M.S., 2004. Evaluation of the genotoxic potential of the *Casearia sylvestris* extract on HTC and V79 cells by the comet assay. Toxicol. In Vitro 18, 337–342.

Majer, B.J., Mersch-Sundermann, V., Darroudi, F., Laky, B., de Wit, K., Knasmüller, S., 2004. Genotoxic effects of dietary and lifestyle related carcinogens in human derived hepatoma (HepG2, Hep3B) cells. Mutat. Res. 551, 153–166.

Maron, D.M., Ames, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat. Res. 113, 173–215.

Miyaji, C.K., Jordao, B.Q., Ribeiro, L.R., Eira, A.F., Colus, I.M.S., 2004. Genotoxicity and anti-genotoxicity assessment of shiitake (*Lentinula edodes* (Berkeley) Pegler) using the Comet assay. Genet. Mol. Biol. 27, 108–114.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nakajima, Y., Tsuruma, K., Shimazawa, M., Mishima, S., Hara, H., 2009. Comparison of bee products based on assays of antioxidant capacities. BMC Complement Altern. Med. 9, 4.

Oberlies, N.H., Burgess, J.P., Navarro, H.A., Pinos, R.E., Fairchild, C.R., Peterson, R.W., Soejarto, D.D., Farnsworth, N.R., Kinghorn, A.D., Wani, M.C., Wall, M.E., 2002. Novel bioactive clerodane diterpenoids from the leaves and twigs of *Casearia sylvestris*. J. Nat. Prod. 65, 95–99.

Sertié, J.A., Carvalho, J.C., Panizza, S., 2000. Antiulcer activity of the crude extract from the leaves of *Casearia sylvestris*. Pharm. Biol. 38, 112–119.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Sousa, F.G., Schneider, N.F.Z., Mendes, C.E., Moura, N.F., Mendes, C.E., Moura, N.F., Denardin, R.B.N., Matuo, R., Mantovani, M.S., 2007. Clastogenic and Anticlastogenic Effect of the Essential Oil from *Casearia sylvestris* Swart. J. Essent. Oil Res. 19, 376–378.

Sterner, D.E., Berger, S.L., 2000. Acetylation of histones and transcription-related factors. Microbiol. Mol. Biol. Rev. 64, 435–459.

Tachino, N., Guo, D., Dashwood, W.M., Yamane, S., Larsen, R., Dashwood, R., 1994. Mechanisms of the in vitro antimutagenic action of chlorophyllin against benzo[a]pyrene: studies of enzyme inhibition, molecular complex formation and degradation of the ultimate carcinogen. Mutat. Res. 308, 191–203.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

Varella, S.D., Pozetti, G.L., Vilegas, W., Varanda, E.A., 2004. Mutagenic activity of sweepings and pigments from a household-wax factory assayed with *Salmonella typhimurium*. Food Chem. Toxicol. 42, 2029–2035.

Vargas, V.M., Motta, V.E., Henriques, J.A., 1993. Mutagenic activity detected by the Ames test in river water under the influence of petrochemical industries. Mutat. Res. 319, 31–45.

Wilkening, S., Stahl, F., Bader, A., 2003. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug Metab. Dispos. 31, 1035–1042.

Zeiger, E., 2007. What is needed for an acceptable antimutagenicity manuscript? Mutat. Res. 626, 1–3.