Chemical and biological characterisation of *Machaerium hirtum* (Vell.) Stellfeld: absence of cytotoxicity and mutagenicity and possible chemopreventive potential

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Abstract

*Machaerium hirtum* (Vell.) Stellfeld (*M. hirtum*) is a plant known as ‘jacarandá-bico-de-pato’ whose bark is commonly used against diarrhea, cough and cancer. The aim of this study was to phytochemically characterise the hydroethanolic extract of this plant, investigate its antimutagenic activities using the Ames test and evaluate its effects on cell viability, genomic instability, gene expression and cell protection in human hepatocellular carcinoma cells (HepG2). Antimutagenic activity was assessed by simultaneous pre- and post-treatment with direct and indirect mutagens, such as 4-nitro-o-phenylenediamine (NPD), mitomycin C (MMC), benzo[a]pyrene (B[a]P) and aflatoxin B₁ (AFB₁), using the Ames test, cytokinesis blocking micronucleus and apoptosis assays. Only 3 of the 10 concentrations evaluated in the MTT assay were cytotoxic in HepG2 cells. Micronucleated or apoptotic cells were not observed with any of the tested concentrations, and there were no mutagenic effects in the bacterial system. However, the Nuclear Division Index and flow cytometry data showed a decrease in cell proliferation. The extract showed an inhibitory effect against direct (NPD) and indirect mutagens (B[a]P and AFB₁). Furthermore, pre- and post-treated cells showed significant reduction in the number of apoptotic and micronucleated cells. This effect is not likely to be associated with the modulation of antioxidant genes, as shown by the RT-qPCR results. Six known flavonoids were identified in the hydroethanolic extract of *Machaerium hirtum* leaves, and their structures were elucidated by spectroscopic and spectrophotometric methods. The presence of the antioxidants apigenin and luteolin may explain these protective effects, because these components can inhibit the formation of reactive species and prevent apoptosis.

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and DNA damage. In conclusion, the M. hirtum extract showed chemopreventive potential and was not hazardous at the tested concentrations in the experiments presented here. Moreover, this extract should be investigated further as a chemopreventive agent.

Introduction

The consumption of medicinal plants is the oldest form of healthcare known to humanity and has been used in all cultures throughout history. This use has evolved from the simplest forms of treatment to the technologically sophisticated forms of industrial manufacturing (1,2). In recent times, many studies have been directed towards the identification of natural products with therapeutic properties (3,4), given the increased use of plant products for cultural, medicinal and social purposes. These natural products can be used directly or may be extracted to identify new bioactive compounds (5). It is essential to evaluate their biological effects to minimise the potential risks to human health (6). Studies concerning genotoxicity and antigenotoxicity may indicate the safety and effectiveness of herbal health products (7) and recommend them as chemopreventive agents.

The Leguminosae family, also known as Fabaceae, is one of the families used for medicinal purposes; this family comprises ~727 genera and 19,327 species located throughout the tropical belt and temperate regions (8). The genus Machaerium is found from southern Mexico to Brazil and as far as northern Argentina in South America, occurring from sea level to 500–900 m and consisting of ~130 species (9) that are predominantly scendent to upright shrubs or lianas.

Flavonoids are the principal chemical compounds of the Machaerium genus (9–11). The extract and isoflavones from the roots of M. aristulatum exhibited giardicidal activity (12). Procyanidin isolated from M. floribundum showed antimicrobial activity against Pseudomonas maltophilia (13). According to Muhammad et al. (14), machaeriol B, obtained from M. multiftonum, demonstrated antimarial activity in vitro against the Plasmodium falciparum W-2 clone. Furthermore, Díaz et al. (15) showed that the Ethanolic extract of M. floribundum displayed significant antioxidant activity, which was confirmed by measuring its capacity to reduce Fe³⁺ to Fe²⁺ (Fenton-type reaction) and cellular viability in fibroblasts.

Machaerium hirtum (Vell.) Steffen commonly occurs in the Brazilian Atlantic Forest and ‘Cerrado’, and it is popularly known as ‘jacarandá-bico-de-pato’, ‘sete casacas’, ‘barreirinho’, ‘bico-de-andorinha’ and ‘jacarandá de espinho’ (16). Its bark is used in folk medicines against diarrhea, cough and cancer (17). According to Ignacio et al. (18), the compounds in M. hirtum include flavanones, alkaloids, triterpenes and steroids. Furthermore, data on their cytotoxic, mutagenic and antimutagenic actions are still lacking. Therefore, because natural products are promising starting points for discovering potentially novel therapeutic agents, the aim of this study was to evaluate the cytotoxic, mutagenic and antimutagenic potential of the hydroethanolic extract of M. hirtum using Salmonella typhimurium in the Ames test and HepG2 cells cultured in vitro. Gene expression and flow cytometry analyses were also used to verify the other possible biological activities of this medicinal plant for its use as a chemopreventive agent.

Materials and methods

Plant material and extract preparation

Machaerium hirtum leaves were collected at São Paulo State, Botucatu City, Rodovia João Hipólito Martins, km 3, Brazil, in December 2012 by Dr Almeida, L.F.R. A voucher specimen of M. hirtum (BOTU 027643) was deposited at the Herbarium of the Department of Botany, Bioscience Institute at the University of São Paulo State, Brazil. The leaves were air-dried and powdered in a mill.

The plant material was extracted with 70% ethanol (EtOH), and the extract was protected from light and percolated at 20 drops/minute, resulting in a hydroalcoholic extract. The solvent was evaporated in vacuo to give a dark residue (170 g, 17% yield).

Fractionation, isolation and identification

The preliminary chemical analysis of the major classes of secondary metabolites from the M. hirtum leaves was performed according to the methods proposed by Matos (19). Thin layer chromatography (TLC) analyses were performed on a silica gel (200 µm; Sorbent Technologies, Norcross, GA, USA), visualised using UV light (254 and 365 nm), and the TLC plates were developed with anisaldehyde/H₂SO₄ and NP/PPEG.

HPLC analytical analysis was performed on a Jasco® HPLC, model PU-2089 (Jasco, Hachioji, Tokyo, Japan), equipped with a MD-2010 Jasco® photodiode array detector and a Phenomenex® Luna (2) RP-18 column (250 × 4.6 mm, 5 µm, Phenomenex, Torrance, CA, USA). The EZChrom Elite Client/Server version 3.1.7 software (Chromatex, Waterloo, UK) was used to acquire and process the chromatographic data.

The plant extract (2.00 g) was dissolved in a mixture of methanol/ water (2:8 v/v) and then partitioned successively with n-hexane (this procedure was repeated three times). The aequous fraction (3.0 g) was fractionated on a column by gel permeation chromatography. The column was packed with Sephadex (2:8 v/v) and then partitioned successively with methanol:water (8:2 v/v) and then successively with water. The column was eluted with the same solvent mixture, and 390 fractions were obtained (500 drops per fraction). The fractions were analysed by TLC, and similar fractions were combined before performing HPLC-RI (RP-18, isocratic, MeOH:H₂O (1:1) and injected directly.

The Electrospray Ionisation Mass Spectrometry (ESI-MS) spectra were obtained using a mass spectrometer (Thermo Scientific, San Jose, CA, USA) in negative mode and were used to identify the major components in the extract. The Xcalibur software (Thermo Scientific) was used during the acquisition and processing of the spectral data. The samples were dissolved in MeOH:H₂O (1:1) and injected directly.
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The $^3$H-NMR and $^{13}$C-NMR experiments used hexadeuterated dimethylsulfoxide (DMSO-d$_6$, Sigma–Aldrich, St Louis, MO, USA) and were performed in a Nuclear Magnetic Resonance 11.7 T (Varian® Inova, Riverside, CA, USA) spectrometer operating at 500 MHz for $^3$H-NMR and 125 MHz for $^{13}$C-NMR, with tetramethylsilane (TMS) as the internal standard.

Chemical reagents

Dimethylsulfoxide (DMSO), nicotinamide adenine dinucleotide phosphate sodium salt (NADP), d-glucose-6-phosphate disodium salt, magnesium chloride, l-histidine monohydrate, d-biotin, 4-nitro-o-phenylenediamine (NPD), sodium azide (SA), mitomycin C (MMC), benz[a]pyrene (B[a]P), aflatoxin B$_1$ (AFB$_1$), 2-aminoanthracone (2-AA), 2-amino-fluorene (2-AF), acridine orange and ethidium bromide were purchased from Sigma Chemical Co. (St Louis, MO, USA). Oxoid Nutrient Broth No. 2 (Oxoid, England) and Difco Bacto Agar (Difco, BD, Franklin Lakes, NJ, USA) were used as the bacterial media.

The metabolic activation mixture (S9 fraction), prepared from Sprague–Dawley rat livers and treated with the polychlorinated biphenyl mixture Aroclor 1254 (500 ppm), was purchased from Molecular Toxicology Inc. (Boone, NC, USA). The MTT salt (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was obtained from Invitrogen (Eugene, OR, USA). d-glucose, magnesium sulfate, citric acid monohydrate, anhydrous dibasic potassium phosphate, sodium ammonium phosphate, monobasic sodium phosphate, dibasic sodium phosphate and sodium chloride were purchased from Merck (Whitehouse Station, NJ, USA).

Ames test

Mutagenic activity was evaluated by the Salmonella/microsome assay, using the Salmonella typhimurium tester strains TA98, TA100, TA97a and TA102, which were kindly provided by Dr B.N. Ames (Berkeley, CA, USA). The test was performed with (+S9) and without (−S9) exogenous metabolism by the pre-incubation method (20).

The S9 mix was freshly prepared before each test. The metabolic activation system consisted of 4% S9 fraction, 1% 0.4 M MgCl$_2$, 1% 1.65 M KCl, 0.5% 1 M d-glucose-6-phosphate disodium, 4% 0.1 M NADP, 50% 0.2 M phosphate buffer and 39.5% sterile distilled water (20). To determine the mutagenic activity, five different concentrations of the M. hirtum extract (0.8–16.0 mg/ml) were diluted in DMSO and assayed. The concentrations of the extract were selected based on a preliminary toxicity test. Toxicity was detected either as a reduction in the number of histidine revertants (His+) or as a thinning of the auxotrophic background lawn. The various tested concentrations of the extract were added to 0.5 ml of 0.2 M phosphate buffer or to 0.5 ml of a 4% S9 mixture with 0.1 ml of bacterial culture and then incubated at 37°C for 20–30 min. Next, 2 ml of top agar was added and the mixture was poured onto a plate containing minimal agar. The plates were incubated at 37°C for 48 h and the His+ revertant colonies were counted manually. All experiments were analysed in triplicate.

The standard mutagens used as positive controls in the experiments without the S9 mix were NPD (10 µg/ml) for TA98 and TA97a, SA (1.25 µg/ml) for TA100 and MMC (0.5 µg/ml) for TA102. In experiments with S9 activation, 2-AA (1.25 µg/ml) was used with TA98, TA97a and TA100, and 2-AF (10 µg/ml) was used with TA102. DMSO (100 µl/ml) served as the negative (solvent) control.

In these tests, the direct mutagens were NPD for S. typhimurium TA98 (10.0 µg/ml) and MMC for S. typhimurium TA102 (0.5 µg/ml). Moreover, B[a]P for S. typhimurium TA98 (1.0 µg/ml) and AFB$_1$ for S. typhimurium TA100 (0.5 µg/ml) were used in the assay with metabolic activation. All of the plates were incubated at 37°C for 48 h, and the number of revertant colonies per plate was counted manually. The entire assay was performed in triplicate. The antimutagenicity results are expressed as percent inhibition, reflecting the ability of the compounds to inhibit the action of the known mutagen. This was calculated as follows:

\[
\text{Inhibition} \% = 100 - \left( \frac{T}{M} \right) \times 100
\]

where $T$ is the number of revertant colonies in the plate containing the mutagen and the compounds and $M$ is the number of revertant colonies in the plate containing only the mutagen (21). No antimutagenic effect was recorded when the inhibition was lower than 25%, a moderate effect for a value between 25 and 40% and strong antimutagenic effects for values greater than 40% (22). Cell viability was also determined for each antimutagenesis experiment to assess the potential bactericidal effect of the mutagens. A substance was considered bactericidal when the bacterial survival was less than 60% of that of the negative control (NC) (22–25).

Cell line and culture conditions

HepG2 cells were kindly provided by the Laboratory of Nutrigenomics of FCFRP of University of São Paulo, Brazil. HepG2 cells were cultured in 10 ml of DMEM Medium (Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS) (Gibco), 1% antibiotic-antimycotic solution (Gibco) and 0.024% sodium bicarbonate (Sigma–Aldrich) at 37°C in 5% CO$_2$. All experiments were conducted between the third and eighth cell passage, and HepG2 cells under these conditions have a cell cycle of ~24 h. All experiments were performed in triplicate with positive (B[a]P – 20 µM) and negative (phosphate-buffered saline, PBS) controls.

Cytotoxicity assay—MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

The MTT assay was used to determine the cytotoxicity of the M. hirtum extract in HepG2 cells using the protocol described by Mosmann (26). Approximately 1.0×10$^4$ cells were plated in each well of 96-well plates in 200 µl of complete culture medium. After 24 h of stabilization, 11 different concentrations of the extract (0.6, 1.2, 2.4, 4.8, 9.7, 19.5, 39.0, 78.1, 156.2, 312.5 and 625 µg/ml, diluted in PBS) were applied to the cells with minimal culture medium (no FBS), with seven wells per concentration, and were sampled at 3, 24, 48, 72 or 96 h. After the appropriate time, the treatments were removed and 100 µl of MTT solution (5 mg/ml) dissolved in complete medium was added to each well and incubated for 4 h. Finally, the medium containing the MTT solution was removed, 200 µl of DMSO was added to each well, and the absorbance was measured with a spectrophotometer (Uniscience, São Paulo, SP, Brazil) at 550 nm. The percentage of viable cells was calculated according to the formula:

\[
\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100
\]

The values of the NC were considered 100% viability and were used to normalise the data.
Micronucleus assay (cytokinesis blocking micronucleus assay)

The micronucleus (MN) assay was performed according to the method described by Fenech (27). First, 0.5 × 10^6 HepG2 cells were placed in each culture flask containing 5 ml of complete medium and stabilised for 24 h. After stabilisation, the cells were treated for 24 h as follows: (i) three concentrations of the *M. bortum* extract (0.6, 2.4 and 9.7 μg/ml) to assess mutagenicity and (ii) one concentration of the extract (2.4 μg/ml) to assess possible antimutagenicity in pre-treatment (treatment with the extract for 24 h and then B[a]P for 24 h), simultaneous treatment (treatment with both the extract and B[a]P for 24 h) and post-treatment (treatment with B[a]P for 24 h followed by the extract for 24 h) protocols. After these treatments, the cells were incubated for 30 h with cytochalasin B (3 μg/ml) (28,29). Positive (B[a]P—20 μM) and negative (PBS) controls were performed for each experiment. The HepG2 cells were washed twice with 5 ml of PBS, trypsinised and centrifuged for 5 min at 174 g/min. Afterwards, the pellet was resuspended in a hypotonic solution (1% sodium citrate, Sigma–Aldrich, at 4°C) containing 30 μl of 25% formaldehyde for 4 min. The cells were centrifuged twice and fixed in 5 ml of a 3:1 methanol:acetic acid solution, transferred to slides and stained with 5% Giemsa (Merk®) for 8 min. Microscopic analysis was performed on a Nikon Eclipse E200 light microscope (Japan). To determine the frequency of micronuclei, 3000 binucleated cells with preserved cytoplasm (1000 cells per replicate) were analysed. The criteria for identification of binucleated cells and micronuclei were those described by Kirsch-Volders et al. (30). The percentage of DNA damage reduction (%R) was obtained from the formula proposed by Waters et al. (31) described below:

\[
%R = \frac{[\% \text{MN B[a]P}] - [\% \text{MN treatment}]}{[\% \text{MN B[a]P}] - [\% \text{MN NC}]} \times 100
\]

where [% MN B[a]P] = the number of micronucleated cells after treatment with benzo[a]pyrene (positive control); [% MN treatment] = the number of micronucleated cells in the different treatments; [% MN NC] = the number of micronucleated cells after treatment with PBS (NC).

The nuclear division index (NDI) was determined using the protocol described by Fenech (27). For each experiment, 500 cells per slide were analysed from the MN experiment, totaling 1500 cells per treatment. To calculate the NDI, we applied the formula proposed by Eastmond and Tucker (32):

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

where M1, M2, M3 and M4 represent the number of cells with one, two, three or four nuclei, respectively, and N is the total number of viable cells analysed.

Apoptosis/necrosis assay

The induction of apoptosis as well as the possible protective effects of *M. bortum* against B[a]P-induced cell death was assessed according to the protocol described by McGahon et al. (33). In total, 1.0 × 10^5 cells were placed in 24-well plates and were treated using the same protocol as for the MN test. After treatment, the cells were trypsinised and 25 μl of the cell suspension was mixed with 1 μl of staining solution (100 μg/ml of acidine orange—AO and 100 μg/ml of ethidium bromide—EB). The homogenate was then placed on a clean slide and covered with a coverslip, and 600 cells per treatment (200 per replicate) were analysed with a Nikon fluorescence microscope, with an excitation wavelength of 515–560 nm and a 590 nm barrier filter. To validate these experiments, a Trypan blue exclusion assay was also performed. The results are presented as the percentage of viable, apoptotic and necrotic cells. The percent of reduced numbers of apoptotic cells (%R) was calculated as described by Waters et al. (31):

\[
%R = \frac{[\% \text{apoptosis B[a]P}] - [\% \text{apoptosis treatment}]}{[\% \text{apoptosis B[a]P}] - [\% \text{apoptosis NC}]} \times 100
\]

Flow cytometry/cell cycle assay

Flow cytometry was performed according to the protocol of Ormerod (34). A total of 1.0 × 10^5 cells was plated in 24-well plates and treated using the same protocol as the MN test. After treatment, the cells were trypsinised, fixed in 2 ml of a 70% ethanol solution and kept on ice until analysis. Immediately before analysis, the cells were centrifuged at 174 g/min for 5 min and resuspended in 400 μl of PBS. Then, 50 μl of RNase solution (1 mg/ml in water) and 50 μl of propidium iodide (400 μg/ml in water) were added and incubated at 37°C for 30 min. The samples were then subjected to fluorescence activated cell sorting (FACS, FACSVantage, Becton Dickinson, Franklin Lakes, NJ, USA); the data were analysed using the Cell Quest software (Becton Dickinson) and cell cycle profiles were examined designed using the Flow Jo software (Tree Star Incorporation, Ashland, OR, USA).

RNA extraction, cDNA synthesis and RT-qPCR

Approximately 0.5 × 10^6 HepG2 cells were stabilised for 24 h in 25 cm² culture flasks with 10 ml of complete DMEM. Then, the culture medium was replaced with PBS or 20 μM B[a]P and the *M. bortum* extract (2.4 μg/ml). Moreover, the protective effect was also evaluated by pre-treating the cells with the negative and positive controls.

Total RNA extraction was performed with the PureLink™ RNA Mini Kit (Ambion - Life Technologies, Foster City, CA, USA) and the RNA was quantified and checked for quality (ratio of absorbance 260/280 between 1.7 and 2.0) by spectrophotometry (NanoDrop 2000C, Thermo Scientific). RNA integrity was also assessed by electrophoresis on a denaturing agarose gel, as described by Aranda et al. (35). The RNA samples were treated with Amplification Grade DNase I (1 U/μl, Invitrogen), according to the manufacturer’s instructions.

The cDNAs were synthesised from 500 ng of total RNA using Oligo-DT® (Invitrogen, Gibco, Grand Island, NY, USA), random primers (Invitrogen) and the SuperScript III enzyme (Invitrogen), according to the manufacturer’s protocol.

The primers for the reference genes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine guanine phosphoribosyl transferase 1 (*HPRT*) and TP53, were designed using Gene Runner Software, version 3.05 (36). The primers for the CAT,
GSR, GPx1, NFE2L2, XPC and CYPIA1 genes were obtained from KGPqStart® SYBR Green Primers (Sigma–Aldrich). The efficiency of each primer was determined by testing serial dilutions of five cDNA concentrations until the best annealing temperature was obtained.

The PCR analyses were performed using the Techne Quantum™ real-time PCR Cycler System with the Platinum® SYBR® Green qPCR SuperMix UGD (Invitrogen, Life Technologies, Van Allen Way, Carlsbad, CA, USA) in a final volume of 10 µl, using 20 pmol of each oligonucleotide primer and 30 ng of the cDNA template. The reaction mixture was subjected to the following amplification program: 95°C for 5 min; 50 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 15 s. A melting curve was also performed, ranging from 50 to 95°C. All reactions were performed in triplicate.

Statistical analysis

The results obtained in the Ames test were analysed with the statistical software package Salanal 1.0 (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, Las Vegas, NV, from Research Triangle Institute, RTP, NC, USA), adopting the Bernstein et al. (37) model. The data (revertants/plate) were assessed by analysis of variance (ANOVA) followed by linear regression. The mutagenic index (MI) was also calculated for each concentration tested, which was the average number of revertants per plate with the test compound divided by the average number of revertants per plate with the negative (solvent) control. A test solution was considered mutagenic when a dose–response relationship was detected and a 2-fold increase in the number of mutants (MI ≥ 2) was observed for at least one concentration (38).

The data obtained from the MTT, CBMN (including NDI) and apoptosis assays were evaluated by ANOVA followed by Tukey’s test. The data obtained from flow cytometry were analysed by ANOVA followed by Dunn’s test. Statistical analyses were performed with GraphPad Prism 5 software (La Jolla, CA, USA). All data are presented as the means ± standard deviation, with a significance level of P ≤ 0.05.

Results

Phytochemical data

The HPLC-PDA chromatographic profile (Figure 1) clearly shows the presence of isoflavones in the UV absorption spectrum, which is characteristic for this class of compounds (41). After isolation, mono- and bi-dimensional NMR spectroscopy was performed to identify the flavonoids as saponarin (apigenin-6-C-β-D-glucopyranosyl-7-O-β-D-glucopyranoside), isolovitin (apigenin-6-C-β-D-glucopyranoside), isoorientin (luteolin-6-C-β-D-glucopyranoside), swertisin (apigenin-7-methoxy-6-C-β-D-glucopyranoside), apigenin-6-C-β-D-glucopyranosyl-8-C-β-D-xylopyranoside and apigenin-8-C-β-D-glucopyranosyl-7-O-β-D-glucopiranoside and using published data for comparison (42,43).

All six compounds were detected in the negative mode of the mass spectrometry analysis (Figure 2). The C-glycosyl flavones were confirmed based on the occurrence of ions produced by the losses of 60, 90 and 120 Da from the cross-ring cleavage of the sugar residues (44).

C-glycosylated flavones have previously been reported in the literature and were identified by mass spectrometry. Kazuno et al. (45) identified C-glycosylated flavones in Rooibos tea using standards and monitoring the characteristic losses at 120 Da. Two flavones, swertisin (apigenin-7-methoxy-6-C-β-D-glucopyranoside) and isovitexin (apigenin-6-C-β-D-glucopyranoside), were identified. Table 1 illustrates the six major constituents in the hydroethanolic extract.

MTT assay

Figure 3 shows the results obtained from the cytotoxicity assay. At all of the treatment times (3, 24, 48, 72 and 96 h), the three highest concentrations tested (156.2, 312.5 and 625 µg/ml) were cytotoxic to HepG2 cells, and the cells treated with 0.6, 2.4 and 9.7 µg/ml showed viability similar to the NC. Therefore, these three concentrations were chosen for the apoptosis, mutagenicity, antimutagenicity and cell cycle kinetic assays.

Figure 1. HPLC-PDA chemical profile of the hydroalcoholic extract from M. hirtum leaves using a Phenomenex® Luna (2) RP18 (250 × 4.6 mm i.d.; 5 µm) column, HPLC (Jasco®), flow 1.0 ml/min. Method: A = H2O, B = MeOH, both with TFA 0.1%. Linear gradient 5–100% of B over 60 min. In the insert, the UV spectra are representative of all peaks. Compounds I and II were not identified.
Assessing the mutagenic and apoptotic effects of *M. hirtum*

Table 2 shows the mean number of revertants/plate (M), the standard deviation (SD) and MI for the *S. typhimurium* strains TA98, TA100, TA102 and TA97a after treatment with the *M. hirtum* extract in the presence (+S9) and absence (−S9) of metabolic activation. *M. hirtum* did not induce an increase in the number of revertant colonies compared to the NC, indicating the absence of any mutagenic activity. These results are consistent with the CBMN assay, where only B[a]P induced a significant increase in the frequency of MNs. This finding indicated that, at the concentrations evaluated, *M. hirtum* has no mutagenic activity (Table 3). In contrast, all concentrations induced a statistically significant decrease in NDI compared to the negative control group (NC). In the Trypan blue assay, the cell viability always remained above 90%, and no difference in cell viability was observed among the groups. In the apoptosis/necrosis assay, only B[a]P induced apoptosis (Figure 4).

Assessing the protective effects of *M. hirtum*

The results obtained in the Ames test for the antimutagenic potential of the *M. hirtum* extract (Table 4) are expressed as the mean number of revertants/plate (M), the standard deviation (SD) and the percent inhibition of the mutagenic activity for a sample containing a mixture of mutagen and extract, compared to the mutagenicity of the mutagen alone. When strain TA98 was used in with NPD, the extract (32% inhibition, 1.0 mg/plate) displayed an antimutagenic effect. However, *M. hirtum* did not reduce MMC-induced mutagenesis in the TA102 strain in the absence of metabolic activation. In experiments with metabolic activation, this botanical species inhibited the AFB1-induced mutation induced in TA100 (84% inhibition, 1.0 mg/plate) and, for strain TA98, the mutagenicity of B[a]P was significantly reduced in a dose-dependent manner from 32 to 85%.

The highest percent inhibition of mutagenicity (85%) was achieved using strain TA98 in the presence of B[a]P. Thus, we decided to continue the experiments using this mutagen.

The concentration of 2.4 μg/ml for the *M. hirtum* extract was chosen for the antimutagenicity assays in the CBMN test, because it did not exhibit a mutagenic effect and it did not induce apoptosis/necrosis. Table 5 shows the data obtained for MNs and NDIs after the different protocols. In all of the treatment protocols used, a protective effect of the *M. hirtum* extract was observed for the B[a]P-induced MNs in HepG2 cells. For NDI, these protocols showed that the extract reversed the antiproliferative effect of B[a]P on HepG2 cells.

Figure 5 shows the data obtained in the apoptosis/necrosis assay. The frequency of apoptotic cells increased significantly after (a) 3 h and (b) 24 h of treatment with B[a]P compared to the controls, and the *M. hirtum* extract showed protective effects on B[a]P-induced cell death. The %R of apoptotic cells after 3 h of treatment was 78, 63 and 78% for the simultaneous, pre- and post-treatments.

Table 1. Flavonoids identified in the *M. hirtum* hydroethanolic extract

<table>
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<tr>
<th>Compound</th>
<th>m/z[M-H]-</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>Name</th>
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<td>593</td>
<td>H</td>
<td>Glucose</td>
<td>H</td>
<td>Glucose</td>
<td>Apigenin-6-C-β-D-glucopyranosyl-7-O-β- d -glucopyranoside</td>
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<td>H</td>
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<td>593</td>
<td>H</td>
<td>H</td>
<td>Glucose</td>
<td>H</td>
<td>Apigenin-8-C-β-d-glucopyranosyl-7-O-β-D-glucopiranoside</td>
</tr>
<tr>
<td>6</td>
<td>563</td>
<td>H</td>
<td>Glucose</td>
<td>Xylose</td>
<td>H</td>
<td>Apigenin-6-C-β-d-glucopyranosyl-8-C-β-D-xylopyranoside</td>
</tr>
</tbody>
</table>
Table 2. Mutagenic activity expressed as the mean and standard deviation of the number of revertants/plate and the mutagenic index (MI) in bacterial strains TA98, TA100, TA102 and TA97a treated with various concentrations of the *M. hirtum* extract, with (+S9) or without (−S9) metabolic activation

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TA98</th>
<th>Treatments</th>
<th>TA100</th>
<th>Treatments</th>
<th>TA102</th>
<th>Treatments</th>
<th>TA97a</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>43±2</td>
<td>20±4</td>
<td>0.0</td>
<td>110±0.6</td>
<td>185±6</td>
<td>170±6</td>
<td>196±10</td>
</tr>
<tr>
<td>1.6</td>
<td>43±8</td>
<td>22±4</td>
<td>0.8</td>
<td>126±8.1</td>
<td>232±14</td>
<td>151±10</td>
<td>230±17</td>
</tr>
<tr>
<td>4.0</td>
<td>51±8</td>
<td>17±3</td>
<td>2.0</td>
<td>110±7.1</td>
<td>225±3</td>
<td>171±3</td>
<td>234±11</td>
</tr>
<tr>
<td>8.0</td>
<td>61±8</td>
<td>16±2</td>
<td>4.0</td>
<td>111±7.1</td>
<td>207±10</td>
<td>172±3</td>
<td>211±2</td>
</tr>
<tr>
<td>12.0</td>
<td>71±0</td>
<td>18±6</td>
<td>6.0</td>
<td>116±0.6</td>
<td>196±9</td>
<td>175±7</td>
<td>192±3</td>
</tr>
<tr>
<td>16.0</td>
<td>50±3</td>
<td>24±6</td>
<td>8.0</td>
<td>100±0.6</td>
<td>161±14</td>
<td>188±5</td>
<td>182±2</td>
</tr>
<tr>
<td>C +</td>
<td>1050±3*</td>
<td>1063±2*</td>
<td>C +</td>
<td>1312±60*</td>
<td>119±5</td>
<td>1720±26*</td>
<td>1168±28*</td>
</tr>
</tbody>
</table>

C + 1050±3* | 1063±2* | C + 1312±60* | 119±5 | 1720±26* | 1168±28* | 1488±18* | 2253±56* |

Figure 3. Percentage of viable HepG2 cells observed after treatment with different concentrations of *M. hirtum* in the MTT assay. The values represent the means ± standard deviation (X ± SD). The results are expressed as the percentage relative to the negative control. NC, negative control—PBS (phosphate-buffered saline). *The values were significantly different from the others. ANOVA followed by Tukey’s test (P ≤ 0.05).
Table 3. The frequency of micronucleated HepG2 cells, the nuclear division index (NDI) and the percentage of micronucleated cells after 24 h of treatment with three different concentrations of the *Machaerium hirtum* extract of and their respective controls

<table>
<thead>
<tr>
<th>Treatments (µg/ml)</th>
<th>Micronucleated cells</th>
<th>% of micronucleated cells</th>
<th>NDI</th>
<th>Replicates</th>
<th>% of inhibition</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicates</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>X ± SD</td>
<td>X ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>16</td>
<td>14</td>
<td>10</td>
<td>13.33 ± 3.06</td>
<td>1.30</td>
</tr>
<tr>
<td>B[a]P</td>
<td></td>
<td>31</td>
<td>24</td>
<td>32</td>
<td>29.00 ± 4.36</td>
<td>2.90</td>
</tr>
<tr>
<td><em>M. hirtum</em> (µg/ml)</td>
<td>0.6</td>
<td>17</td>
<td>15</td>
<td>15</td>
<td>15.67 ± 1.16</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>13</td>
<td>12</td>
<td>11</td>
<td>12.00 ± 1.00</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>9.7</td>
<td>16</td>
<td>13</td>
<td>15</td>
<td>14.67 ± 1.53</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
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<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>4.00</td>
<td>4.00</td>
</tr>
</tbody>
</table>

A total of 3000 binucleated cells per treatment was counted to score the MN cells and 1500 cells were counted for the nuclear division index (NDI). Negative control (PBS: phosphate-buffered saline); positive control (B[a]P 20 µM). The values represent the means ± standard deviation (X ± SD).

*The values are significantly different from the others in the same column. ANOVA followed by Tukey’s test (P ≤ 0.05).*

Table 4. Antimutagenic activity expressed as the mean and standard deviation of number of revertants and the percent inhibition by *M.hirtum* of direct (−S9) and indirect (+S9) mutagens

<table>
<thead>
<tr>
<th>Number of revertants (X ± SD)/plate and % of inhibition</th>
<th>TA98 (−S9)</th>
<th>TA102 (−S9)</th>
<th>TA98 (+S9)</th>
<th>TA100 (+S9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. hirtum</em> + NPD</td>
<td>mg/plate</td>
<td>mg/plate</td>
<td>mg/plate</td>
<td>mg/plate</td>
</tr>
<tr>
<td>NPD</td>
<td>752 ± 38</td>
<td>1731 ± 86</td>
<td>267 ± 34</td>
<td>859 ± 103</td>
</tr>
<tr>
<td>DMSO</td>
<td>32 ± 2</td>
<td>393 ± 19</td>
<td>173 ± 3</td>
<td>122 ± 26</td>
</tr>
<tr>
<td>0.25</td>
<td>571 ± 28 (24±)</td>
<td>1709 ± 24 (14±)</td>
<td>182 ± 21 (32±)</td>
<td>559 ± 64 (35±)</td>
</tr>
<tr>
<td>0.5</td>
<td>536 ± 26 (29±)</td>
<td>1569 ± 79 (9±)</td>
<td>156 ± 34 (42±)</td>
<td>269 ± 53 (69±)</td>
</tr>
<tr>
<td>1.0</td>
<td>513 ± 37 (32±)</td>
<td>1706 ± 59 (13±)</td>
<td>10 ± 18 (74±)</td>
<td>134 ± 18 (84±)</td>
</tr>
<tr>
<td>2.0</td>
<td>523 ± 35 (30±)</td>
<td>1738 ± 61 (-)</td>
<td>2 ± 19 (80±)</td>
<td>162 ± 45 (81±)</td>
</tr>
<tr>
<td>4.0</td>
<td>524 ± 19 (30±)</td>
<td>1892 ± 93 (-)</td>
<td>4 ± 17 (85±)</td>
<td>144 ± 34 (83±)</td>
</tr>
</tbody>
</table>

AFB1, aflatoxin B1 (0.5 µg/plate); B[a]P, benzo[a]pyrene (1.0 µg/plate); M ± SD, mean and standard deviation; NPD, 4-nitro-o-phenylenediamine (10.0 µg/plate); MMC, mitomycin C (0.5 µg/plate).

*No antimutagenic effect (< 25% inhibition).*

*Moderate effect (25–40% inhibition).*

*Strong antimutagenic effect (> 40% inhibition).*

respectively. After 24 h, the protective effect was 120% for the pre- and post-treatments. In the simultaneous treatment, the result was statistically equal to the positive control. Therefore, the best results were observed after 24 h in the pre- and post-treatments (Figure 5). There was no difference in the number of necrotic cells between the different treatments.
Figure 5. Percentage (%) of viable, apoptotic and necrotic HepG2 cells after (a) 3 h or (b) 24 h of treatment with the *Machaerium hirtum* extract and B[a]P (20 μM) using pre-treatment (PRE), simultaneous treatment (SIM) and post-treatment (POS) protocols after differential staining with acridine orange and ethidium bromide in the apoptosis/necrosis assay. The values represent the means ± standard deviation (X ± SD) of experiments performed in triplicate; a total of 600 cells was analysed per treatment. NC, negative control—PBS (phosphate buffered saline); PC, positive control—B[a]P (20 μM). *The values were significantly different from the others using the same parameter. ANOVA followed by Tukey’s test (P ≤ 0.05).

Table 5. The frequency of micronucleated HepG2 cells, the nuclear division index (NDI) and the percentage of micronucleated cells after 24 h of treatment with the *Machaerium hirtum* extract and B[a]P (20 μM) in simultaneous, pre- and post-treatment protocols with their respective controls.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Micronucleated cells</th>
<th>% of micronucleated cells</th>
<th>%R</th>
<th>NDI</th>
<th>Replicates</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicates</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>X ± SD</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>X ± SD</td>
</tr>
<tr>
<td>Simultaneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PBS</td>
<td>16 10 11</td>
<td>12.33 ± 3.21</td>
<td>1.23</td>
<td>1.518</td>
<td>1.524</td>
<td>1.522</td>
<td>1.521 ± 0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>26 27 30</td>
<td>27.67 ± 2.08</td>
<td>2.77</td>
<td>1.366</td>
<td>1.378</td>
<td>1.414</td>
<td>1.386 ± 0.025</td>
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<td></td>
</tr>
<tr>
<td><em>M.hirtum</em> + B[a]P</td>
<td>20 16 15</td>
<td>17.00 ± 2.63</td>
<td>1.70</td>
<td>1.726</td>
<td>1.626</td>
<td>1.456</td>
<td>1.603 ± 0.136</td>
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<td></td>
</tr>
<tr>
<td>Pre-treatment</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>11 12 10</td>
<td>11.00 ± 1.00</td>
<td>1.10</td>
<td>1.592</td>
<td>1.490</td>
<td>1.518</td>
<td>1.533 ± 0.053</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>23 23 26</td>
<td>24.00 ± 1.73</td>
<td>2.40</td>
<td>1.438</td>
<td>1.460</td>
<td>1.400</td>
<td>1.433 ± 0.030</td>
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<td></td>
</tr>
<tr>
<td><em>M.hirtum</em> + B[a]P</td>
<td>13 17 14</td>
<td>14.67 ± 2.08</td>
<td>1.47</td>
<td>1.338</td>
<td>1.498</td>
<td>1.502</td>
<td>1.446 ± 0.094</td>
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<tr>
<td>Post-treatment</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>11 12 10</td>
<td>11.00 ± 1.00</td>
<td>1.10</td>
<td>1.592</td>
<td>1.490</td>
<td>1.518</td>
<td>1.533 ± 0.053</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>26 29 25</td>
<td>26.67 ± 2.08</td>
<td>2.67</td>
<td>1.416</td>
<td>1.438</td>
<td>1.390</td>
<td>1.415 ± 0.024</td>
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<td></td>
</tr>
<tr>
<td>B[a]P + <em>M.hirtum</em></td>
<td>15 15 16</td>
<td>15.33 ± 0.58</td>
<td>1.53</td>
<td>1.434</td>
<td>1.484</td>
<td>1.446</td>
<td>1.455 ± 0.026</td>
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</tbody>
</table>

A total of 3000 binucleated cells per treatment was counted for the MN frequency and a total of 1500 cells was counted for the nuclear division index (NDI). Negative control, PBS (phosphate-buffered saline); positive control, B[a]P (20 μM). The values represent the means ± standard deviation (X ± SD). Values with the same letter did not differ from each other. ANOVA followed by Tukey’s test (P ≤ 0.05).
DNA content analysis

The results of the cell cycle assay performed by flow cytometry using staining with propidium iodide are shown in Figure 6. In Figure 6A, when the data of the NC were compared with that of the M. hirtum extract, the extract significantly decreased the cell population in G1 phase at the three concentrations tested. At a concentration of 0.6 μg/ml, there was a significant increase in the population of cells in S phase, and at concentrations of 0.6 and 2.4 μg/ml, there was an increase in the population of cells in G2/M phase. These results demonstrate an arrest of the cell cycle that was particularly promoted by the lowest concentration tested (0.6 μg/ml). B[a]P also changed the cell cycle when compared to the NC.

The evaluation of the protective effects (Figure 6B) shows that the B[a]P-induced alterations in the percentage of cells in each phase of the cell cycle were prevented in the pre-treatment group, and these differences were reinforced in the post-treatment group.

Table 6. Gene expression in HepG2 cells analysed by real-time polymerase chain reaction (RT-qPCR) after 24 h of treatment with M. hirtum (2.4 μg/ml) alone or in combination with benzo[a]pyrene (B[a]P) in the pre-treatment protocol along with their respective control groups

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>1.425</td>
<td>1.101</td>
<td>1.281</td>
<td>0.683</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>1.215</td>
<td>1.224</td>
<td>1.106</td>
<td>0.703</td>
</tr>
<tr>
<td>GPX1</td>
<td>0.627</td>
<td>0.932</td>
<td>1.106</td>
<td>0.703</td>
</tr>
<tr>
<td>GSR</td>
<td>0.896</td>
<td>0.951</td>
<td>0.985</td>
<td>0.703</td>
</tr>
<tr>
<td>XPC</td>
<td>1.081</td>
<td>0.858</td>
<td>1.389</td>
<td>0.780</td>
</tr>
<tr>
<td>TP53</td>
<td>0.801</td>
<td>0.983</td>
<td>0.894</td>
<td>0.869</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>5.096</td>
<td>1.425</td>
<td>6.961</td>
<td>1.286</td>
</tr>
</tbody>
</table>

Differences in gene expression compared to the respective negative control group (NC) in the mutagenicity protocol and to the positive control group (B[a]P/20 μM) in protective effects protocol. All experiments were performed in triplicate. Values in bold were considered significantly different compared to its respective control group \( \text{P} < 0.05 \) and fold change (FC) higher than 2.

CAT, catalase; NFE2L2, nuclear factor erythroid 2-like-2; GPX1, glutathione peroxidase 1; GSR, glutathione reductase; XPC, Xeroderma Pigmentosum complementation group C; TP53, tumor protein p53; CYP1A1, cytochrome P450, Family 1, Subfamily A, polypeptide 1.

DNA content analysis

The results of the cell cycle assay performed by flow cytometry using staining with propidium iodide are shown in Figure 6. In Figure 6A, when the data of the NC were compared with that of the M. hirtum extract, the extract significantly decreased the cell population in G1 phase at the three concentrations tested. At a concentration of 0.6 μg/ml, there was a significant increase in the population of cells in S phase, and at concentrations of 0.6 and 2.4 μg/ml, there was an increase in the population of cells in G2/M phase. These results demonstrate an arrest of the cell cycle that was particularly promoted by the lowest concentration tested (0.6 μg/ml). B[a]P also changed the cell cycle when compared to the NC.

The evaluation of the protective effects (Figure 6B) shows that the B[a]P-induced alterations in the percentage of cells in each phase of the cell cycle were prevented in the pre-treatment group, and these differences were reinforced in the post-treatment group.
Gene expression
The gene expression analysis using RT-qPCR (Table 6) showed that M. hirtum (2.4 µg/ml) did not alter the expression of genes related to oxidative stress (CAT, GSR, GPX1, NFE2L2), drug metabolism (CYP1A1), tumour suppression (TP53) or DNA repair (XPC). In the pre-treatment protocol, no statistically significant increases were observed. However, B[a]P alone and in association with the M. hirtum extract positively modulated the expression of CYP1A1.

Discussion
Natural plants have been used to prevent and to treat various diseases for thousands of years (46), and the plant-derived compounds can provide additional beneficial effects, such as activation of the immune system, anti-inflammatory activity (18) and antimutagenic effects (47). Dietary recommendations related to the safe consumption of plants with different chemical compositions are of great importance; however, the biological activities of many plant extracts remain to be explored.

In the present study, the phytochemical analysis showed that the M. hirtum hydroethanolic extract is rich in mainly C-glycoside flavonoids. The flavonoid compounds may appear in both the nonglycosylated and glycosylated forms. The majority of the C-glycosylated flavonoids from the M. hirtum extract are from the apigenin family: saponarin (apigenin-6-C-β-D-glucopyranosyl-7-O-β-D-glucopyranoside) and isovitexin (apigenin-6-C-β-D-glucopyranoside). The luteolin family is represented by isoorientin (luteolin-6-C-β-D-glucopyranoside). Two of the identified flavones, swertisin (apigenin-7-methoxy-6-C-β-D-glucopyranoside) and isovitexin (apigenin-6-C-β-D-glucopyranoside), were also previously identified in the M. hirtum extract from by Igoato et al. (18). According to Manach et al. (48), these glycoside flavonoids, depending on their structure, may differ in their bioavailability in humans, becoming more or less available than aglycone.

Analysis of the cytotoxic effects is often the first test to be performed when a new vegetal extract or plant compound is first analysed. The data from the present study show that only the higher concentrations of the M. hirtum extract (156.2, 312.5 and 625 µg/ml) decreased the viability of HepG2 cells. This fact highlights the importance of assessing different concentrations to classify the cytotoxicity of an extract. Jeremic et al. (49) demonstrated the cytotoxic effects of Sideritis scardica extracts, such as cell cycle arrest and apoptosis, and suggested that this activity was mediated by the main chemical constituents, apigenin and luteolin, the same main compounds observed in M. hirtum.

Previous studies have indicated that luteolin is beneficial for preventing tumour progression (50) and has pro-apoptotic activity in tumour cells (51). Although M. hirtum extract is rich in luteolin, it did not induce apoptosis, which is likely because the previous studies used isolated luteolin in higher concentrations. However, in the present study, luteolin was a component of the extract and was therefore present in a minor amount and interacted with the other constituents.

Different concentrations of the M. hirtum extract did not induce chromosomal alterations (CBMN test) in metabolically active human cells or point mutations (Ames test) in different strains of S. typhimurium in the absence and in the presence of the metabolically active fraction (59). The major flavonoid of the M. hirtum extract, apigenin, was previously evaluated and was also not mutagenic in strains TA98 and TA100 of S. typhimurium by the Ames test and MN test in CD1 mice (52). The absence of mutagenic effects by the extract is a positive step towards ensuring its safe use in medicine.

In the Ames test, the M. hirtum extract exhibited an antimutagenic effect only against NPD-induced frameshift mutations in the TA98 strain, with the highest % of inhibition at a concentration of 1.0 mg/plate (32%). The exact mechanism by which the M. hirtum extract showed antimutagenicity is not clear. There are several possibilities of the extract provide protection to the bacterial genome against this direct mutagen such as it may (i) have promoted the rapid elimination of NPD from the bacteria before the interaction with the DNA, which may be mediated by facilitating or stimulating the transmembrane export system in bacteria (23,24); (ii) interfere with the uptake of the mutagen; (iii) inactivate directly the mutagen by complex formation with the ingredients present in the extract.

The observed antimutagenic effect was more evident when with indirect mutagens, such as the environmental procarcinogen B[a]P and a fungal toxin AFB1. M. hirtum reduced the frequency of AFB1-induced mutations by 84%, which may have been mediated through the inhibition or inactivation of the exo-epoxide form of AFB1, (in the S9 fraction), preventing the formation of adducts (53). Moreover, the decrease in B[a]P-induced DNA adduct formation may have occurred through the interaction of the extract with the reactive intermediates of B[a]P, such as B[a]P-7,8-dihydriodiol-9,10-epoxide, that alkylate nucleosides on DNA molecules or interfere with the microsomal enzyme activities that are required to produce the final carcinogen (25,54). The highest observed percent inhibition of mutagenicity (85%) achieved with the M. hirtum extract, occurred in the TA98 strain in the presence of B[a]P. Thus, we decided to continue the investigation of the chemoprotective effects of the M. hirtum extract against this mutagen.

The flavonoid apigenin exhibited antimutagenic activities in a variety of techniques in vivo and in vitro, such as the Ames test (52,53), MN test (52,56), comet assay (57) and enzymatic parameters (58). In a recent study, luteolin did not show mutagenicity in the Ames test (25). Moreover, the Ames test was used to study the antimutagenicity of 10 flavonoids against different mutagens, and luteolin was the only compound that exhibited a protective effect against all of the mutagens (22).

B[a]P exerts its cytotoxic and mutagenic effects by generating reactive species, which, when produced in excess, result in cell death by apoptosis (59,60). Additionally, B[a]P induces DNA adducts by covalent bonding (61). According to Niestroy et al. (62), compounds that occur naturally in plants, such as flavonoids, may interfere as agonists or antagonists in the metabolic pathways of B[a]P to modulate its action. In this study, we demonstrated that M. hirtum decreased the B[a]P-induced mutagenicity and apoptosis. These results suggest an antioxidant effect for the extract that could be related to inhibiting the B[a]P metabolism, because protective effects were observed in the pre-treatment group. M. hirtum could also activate cellular repair, as shown by the protective effects in the post-treatment group. To confirm these hypotheses, we analysed the gene expression of NFE2L2, which encodes a transcription factor that acts in the promoter regions of genes involved in xenobiotic detoxification. Genes related to oxidative stress, CAT, GSR and GPX1, were also evaluated. TP53, XPC and CYP1A1 were tested to analyse the influence of the M. hirtum extract on DNA repair and B[a]P metabolism.

Khan et al. (58) demonstrated that apigenin suppressed B[a]P metabolism in vivo by inhibiting phase I enzymes and inducing some phase II enzymes, decreasing the availability of reactive carcinogenic metabolites. The fact that apigenin is one of the major components of the M. hirtum extract suggests that this component may be one of the components responsible for the protective effects observed in the
MN test in the present study. However, this protective effect does not appear to be related to the alterations in gene expression, because the M. hirtum extract did not modulate the antioxidant genes. B[a]P alone positively modulated the expression of the CYP1A1 gene, the main catalytic enzyme responsible for the metabolism of genotoxic compounds, such as B[a]P-7,9-dihydrodiol-9,10-epoxide (63). This result suggests that M. hirtum could decrease B[a]P activation by decreasing the formation of its reactive metabolites and decrease the B[a]P mutagenicity in the MN assay. This antimutagenic effect may also be related to an interaction between the constituents of the extract (administered previously) and B[a]P by inactivating it and decreasing the MN formation and apoptosis (58, 54).

There are no data about the effects of M. hirtum on gene expression; however, its major constituents, apigenin and luteolin, have been tested alone. As observed by Huang et al. (63) in primary rat hepatocytes, the mechanism of action of these two flavonoids is to activate NFE2L2 and increase the expression of GSR. Contradictory results were obtained by Tang et al. (66), demonstrating that luteolin inhibited the expression of NFE2L2 and caused the depletion of GSH levels in A549 lung cancer cells. Apigenin can act by increasing the expression of repair genes, such as XPC, in skin keratinocyte cells (HaCaT) in vitro (67). In the present study, no modifications in the expression of NFE2L2 or XPC were observed in HepG2 cells, likely because apigenin and luteolin were present at low concentrations in the M. hirtum extract or they interacted with other constituents of the extract. The results obtained in our study suggest that the absence of alterations in NFE2L2 expression blocked the induction of other antioxidant genes, such as CAT, GPX1 and GSR.

The analysis of the cell cycle by flow cytometry showed that the M. hirtum extract arrested HepG2 cells in G2/M phase, as previously demonstrated in studies with luteolin (68). According to the same authors, this arrest may be due to the ability of luteolin to inhibit the DNA topoisomerase I (Topo-I) enzyme, blocking the replication process and resulting in discontinued G2/M phase. Furthermore, Zhang et al. (69) observed the same phenomenon of cell cycle arrest in G2/M phase in other cell types with structurally related flavonoids, such as apigenin, quercetin, kaempferol and myricetin. These results added to the MTT test, where high and low concentrations of M. hirtum extract presented difference on cell viability, encourage the recommendation of use of low doses of extract for chemoprevention purposes and high doses for chemotherapy purposes.

In conclusion, our study demonstrated that the M. hirtum extract arrested HepG2 cells to G2/M phase, as previously demonstrated in studies with luteolin (68). According to the same authors, this arrest may be due to the ability of luteolin to inhibit the DNA topoisomerase I (Topo-I) enzyme, blocking the replication process and resulting in discontinued G2/M phase. Furthermore, Zhang et al. (69) observed the same phenomenon of cell cycle arrest in G2/M phase in other cell types with structurally related flavonoids, such as apigenin, quercetin, kaempferol and myricetin. These results added to the MTT test, where high and low concentrations of M. hirtum extract presented difference on cell viability, encourage the recommendation of use of low doses of extract for chemoprevention purposes and high doses for chemotherapy purposes.

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References

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