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# Chemical composition and *in vitro* chemoprevention assessment of *Eugenia jambolana* Lam. (Myrtaceae) fruits and leaves

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*Chemical compounds:*
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Myricitrin (PubChem CID: 5281673)**2**
Malvidin-3-*O*-gentibioside (PubChem CID: 44256980)**3**
Delphinidin-3-*O*-gentibioside (PubChem CID: 44256919)**4**
Cyanidin-3-*O*-gentibioside (PubChem CID: 44256722) **5**
Petunidin-3-*O*-gentibioside (PubChem CID: 44256956)**6**
Delphinidin-3-*O*-glucoside (PubChem CID: 443650)**7**
Cyanidin-3-*O*-glucoside (PubChem CID: 44256715)**8**
Petunidin-3-*O*-glucoside (PubChem CID: 443651)**9**
Malvidin-3-*O*-glucoside (PubChem CID: 443652)**10**

ABSTRACT

*Eugenia jambolana* has been used as an antioxidant, anti-inflammatory and antidiabetic. This study performed a chemical and chemopreventive screening of extracts and fractions from leaves and fruits from *Eugenia jambolana*. The purification of the crude extract from leaves afforded two known flavonoids, tricetin-4′-*O*-α-L-rhamnopyranoside (**1**), and myricitrin (**2**), whereas the purification of crude extract from fruits afforded one anthocyanin, malvidin-3-*O*-gentibioside (**3**). Additionally, seven anthocyanins were tentatively identified. The antigenotoxicity results showed that post-treatment with extracts and fractions mitigated the damage induced by hydrogen peroxide. In assessing the antimutagenicity, the samples were able to reduce the frequency of micronucleus formation for both pre-treatment and post-treatment. Also, extracts and fractions promoted the induction of quinone reductase activity. Such extracts and fractions were able to induce detoxifying enzymes and reverse DNA damage, an essential balance for its applicability as a chemopreventive agent.



*Abbreviations:* CEL, crude extract leaves; CEF, crude extract fruits; HL, hexane fraction leaves; HF, hexane fraction fruits; EtOAcL, ethyl acetate fraction leaves; EtOAcF, ethyl acetate fraction fruits; BuL, *n*-buthanolic fraction leaves; BuF, *n*-buthanolic fraction fruits; HAL, hydroalcoholic fraction leaves; HAF, hydroalcoholic fraction fruits; NQO1, quinone reductase; HepG2, human hepatoma cell line.

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## 1. Introduction

*Eugenia jambolana* Lam. or *Syzygium cumini* Skeels., a plant of the Myrtaceae family, is commonly known as jambolão in Brazil, jamun in India, and black plum in Europe (Ayyanar, Subash-Babu, & Ignacimuthu, 2013). In traditional medicine, especially in Indian Ayurveda medicine, seeds and fruit pulp are used in the treatment of diabetes (Ayyanar et al., 2013). However, Tong, Wang, Waisundara, and Huang (2014) also reported the use of the herbal tea from its bark for the treatment of diabetes. Ayyanar et al. (2013) observed the antidiabetic effects of various parts of *E. jambolana* in diabetic animal models and patients. Several studies have described pharmacological actions of *E. jambolana* extracts, including: antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antifertility, antidiabetic, gastroprotective, hepatoprotective, hypolipidemic, cardioprotective, anti-diarrheal, anti-allergic, antipyretic, antineoplastic, neuropsychopharmacological, chemopreventive, radioprotective, and anticlastogenic (Aqil, Jeyabalan, Munagala, Singh, & Gupta, 2016; Ayyanar & Subash-Babu, 2012; Baliga, Bhat, Baliga, Wilson, & Palatty, 2011; Muruganandan et al., 2001; Rajasekaran et al., 1988; Sanches et al., 2016; Sharma, Nasir, Prabhu, & Murthy, 2006; Sharma, Siddiqui, Kumar, Ram, & Chaudhary, 2013; Chagas, França, Malik, & Paes, 2015).

The main chemical compounds reported for *E. jambolana* which may associated to health benefits are terpenes, flavonoids, and phenolic acids, which are isolated from leaves, seeds, flowers, stem barks, and fruits (Baliga et al., 2011). The leaves contain triterpenes: β-sitosterol, betulinic acid, crategolic acid, and oleanolic acid (Rajasekaran et al., 1988; Gupta & Sharma, 1974), phenolic acids: gallic and ellagic acids and their derivatives (Mahmoud, Marzouk, Moharram, El-Gind, & Hassan, 2001; Sanches et al., 2016), and flavonoids: quercetin, myricetin, myricetin derivatives, myricitrin, kaempferol, and kaempferol, (Latief et al., 2015; Mahmoud et al., 2001; Sanches et al., 2016). The fruits contain flavonoids, specifically, anthocyanins, including: cyanidin, delphinidin, petunidin, malvidin, and peonidin-mono and diglucosides, which are responsible for their purple color (Li, Zhang, & Seeram, 2009; Li et al., 2009; Reynertson, Yang, Jiang, Basile, & Kennely, 2008; Sharma, Gupta, Singh, Bansal, & Sing, 2016; Zhang, Seeram, Lee, Feng, & Heber, 2008; Brito et al., 2007). These anthocyanins have been related to the chemopreventive effects of *E. jambolana* fruit extracts (Li et al., 2009).

A large number of studies have shown that diets rich in fruits and vegetables can reduce the risk for developing chronic diseases, such as cardiovascular disease, cancer and diabetes (Lilamand et al., 2014; Yang et al., 2011). Foods and fruits which contain high levels of antioxidants, such as polyphenols, phenolic acids, carotenoids, flavonoids and anthocyanins, may reduce the levels of reactive oxygen species in mammalian organisms and prevent DNA damage and mutations that initiate tumor progression (Zhang et al., 2008; Hogan et al., 2010). Therefore, it is hypothesized that a variety of compounds identified in plants may have antimutagenic and antigenotoxic activities (Cariño-Cortés et al., 2007).

The micronucleus test and comet assay are widely used for identifying both genotoxic and chemopreventive agents because they are sensitive, easy to implement, and can be performed with various cell lines (Lamy, Schmitz, Krumbain, & Mesch Sunderman, 2011). The difference between the two tests is primarily due to variations in the type of DNA damage; the micronucleus test detects irreparable injuries that manifest as chromosomal aberrations or aneugenic effects, while the comet assay detects primary DNA lesions with repairable damage (Salvadori, Ribeiro, & Natarajan, 1993; Scolastici et al., 2008). The quinone reductase (NQO1) is a microsomal phase 2 enzyme, which participates in detoxification reactions within the body, and therefore, the ability to promote its induction is considered indicative of chemopreventive activity when testing new drugs (Yang & Liu, 2009). The human hepatoma cell line (HepG2) is easy to handle, may retain many of the characteristics of the liver parenchymal cells, and contains various enzymes responsible for the bio-activation of several xenobiotic-metabolizing agents (Salvadori et al., 1993).

The aim of the present study was to investigate the chemical composition of extracts and fractions from leaves and fruits of *E. jambolana* using chromatographic and spectroscopic techniques such as HPLC-PDA-MS/MS and NMR. In additional, the genotoxic, antigenotoxic, mutagenic, and antimutagenic effects, as well as the quinone reductase capacity of extracts and fractions on HepG2 cells by using the comet assay and micronucleus test were investigated.

## 2. Material and methods

### 2.1. General experimental procedures

All solvents used for extraction, purification, and isolation were of ACS reagent grade and were purchased from JT Baker® (Center Valley, PA, USA). Additionally, we used ultrapure water (Millipore system, Billerica, USA). Chloridric acid (P.A.) was obtained from JT Baker® (Center Valley, PA, USA) and trifluoroacetic acid (chromatographic grade) was obtained from Tedia (Rio de Janeiro, RJ, Brazil). Deuterated solvents (dimethylsulphoxide (DMSO-$d_6$), methanol ($CD_3OD$), and trifluoroacetic acid (TFA-d)) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Chromatographic column analysis was performed using reverse phase silica RP-18 (50–60 μm) purchased from Machery-Nagel (Macherey-Nagel GmbH & Co. KG, Duren, DEU) and the 250 μm thin-layer chromatography (TLC) plates were from Analtech, Inc. (Newark, DE, USA). NMR spectra were recorded on Varian Inova 500® Varian VRX instruments (Varian, Palo Alto, CA, USA), and Bruker Avance III 600® (Bruker Biospin GMBH, Rheinstetten, Germany). HPLC-PDA analyses were carried out on a Shimadzu HPLC (Kyoto, Japan), while HPLC-PDA-MS/MS analyses were carried out on a Shimadzu HPLC (Kyoto, Japan) connected in series to a mass spectrometer (MS/MS) from Bruker Daltonics (Massachusetts, USA). The MS system used was a quadrupole time-of-flight instrument (UltrO-TOF-Q, Bruker Daltonics, Billerica, Massachusetts, USA). The HepG2 cells used in the comet assay and cytotoxicity assay by Sulforhodamine B and Hepa 1c1c7 cells used in quinone reductase induction assay were cultivated in Dulbecco's Modified Eagle Medium and Alpha Minimum Essential Medium Eagle, respectively, supplemented with penicillin and streptomycin, sodium bicarbonate, kanamycin Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and fetal bovine serum (Cultilab, Campinas, SP, Brazil). Sodium chloride, EDTA, Tris, triton-X 100, DMSO, sodium

hydroxide solutions, normal melting point agarose and low melting point agarose employed in the comet assay were obtained from Sigma-Aldrich Chemical Co. Cytochalasin B, methanol, acetic acid and potassium chloride used in the micronucleus assay and trypsin used in cell experiments were also obtained from Sigma-Aldrich Chemical Co. The comet and micronucleus assays were performed using a Nikon microscope model ECLIPSE 50i, coupled with a Nikon camera model DS-Ri1 (Nikon Instruments Inc., Japan). Sulforhodamine B and quinone reductase induction assays were analyzed in a Bio-Rad microplate reader model iMark (Bio-Rad Laboratories, Inc., CA, USA).

### 2.2. Plant Material

Leaves and fruits of *Eugenia jambolana* were collected in Araraquara, Sao Paulo State, Brazil - 21°47′24″ Lat. S and 48°10′12″ Log. W, in March 2008 and a voucher specimen (SJRP 19586) was deposited at Herbarium of Sao José do Rio Preto.

### 2.3. Extraction and isolation

Leaves of *E. jambolana* were dried at 45 °C, ground and extracted exhaustively at room temperature using ethanol. The ethanol solution was concentrated under reduced pressure at 40 °C to give the crude extract of leaves (CEL, 26 g). An aliquot of CEL was dissolved in methanol:water (8:2, v/v) and submitted to partition using hexane, ethyl acetate, and *n*-butanol to yield hexane fraction (HL1, 4.6 g), ethyl acetate fraction (EtOAcL, 4.1 g), *n*-butanol fraction (BuL, 3.1 g), and hydroalcoholic fraction (HAL, 5.5 g), which were analyzed by TLC. BuL and HAL chemical profiles disclosed the presence of phenolic compounds, especially flavonoids, and were then selected for further fractionation. BuL fraction was submitted to RP-18 column chromatography using MeOH:$H_2O$ and gradient elution to yield 21 sub-fractions (BuL1 - BuL21). BuL14 was subjected to preparative HPLC purification using a RP-18 column and MeCN:$H_2O$ (1:9) under isocratic elution to afford compound **1** (2.0 mg). Fraction HAL was also submitted to RP-18 column chromatography using MeOH:$H_2O$ under gradient elution to yield 15 sub-fractions (HAL1 - HAL15). HAL6 was re-submitted to RP-18 column chromatography using MeOH:$H_2O$ and gradient elution to yield 11 sub-fractions. HAL6_7 was subjected to preparative HPLC using a RP-18 column and MeCN:$H_2O$ (15:85) under isocratic elution to afford compound **2** (4.1 mg).

Fresh fruits of *E. jambolana* were deseeded and the anthocyanins were extracted using methanol (1% HCl) (500 mL, 5 min, 3×) under constant agitation. The mixture was centrifuged at 10,000*g* for 10 min at 4 °C, and the supernatant was then concentrated under reduced pressure at 40 °C to yield the crude extract of fruits (CEF, 420 g) (Hong & Wrolstad, 1990; Seeram, Schutzki, Chandra, & Nair, 2002). An aliquot of CEF was dissolved in methanol (0.1% HCl):water (0.1% HCl) (9:1) and submitted to partition with organic solvents to yield hexane fraction (HF, 17 mg), ethyl acetate fraction (EtOAcF, 5.0 g), *n*-butanol fraction (BuF, 0.57 g), and hydroalcoholic fraction (HAF, 17.5 g). HAF (5 g) which purple color evidenced a high yield in anthocyanins was subjected to reverse phase RP-18 column chromatography using MeOH (0.1% HCl):$H_2O$ (0.1% HCl) and linear gradient to yield sub-fractions: F-I (1.071 mg), F-II (2.845 mg), F-III (374 mg), F-IV (34.2 mg), F-V (39.8 mg), F-VI (37.2 mg), F-VII (33.3 mg), F-VIII (30.5 mg), F-IX (31.2 mg), and F-X (36.9 mg). All sub-fractions were analyzed by TLC, and sub-fraction F-X was shown to contain one isolated anthocyanin, identified as compound **3**.

CEL and CEF and all fractions were solubilized in DMSO and tested in biological assays.

Compound **3**: *Malvinidin-3-O-gentiobioside*: $^1$H NMR (500 MHz, $CD_3OD$:$CF_3COOD$ (19:1)): $\delta$ 9.17 (1H; s; H-4), 8.04 (2H; s; H-2′ and H-6′), 7.09 (1H; d; $J$ = 2.0 Hz; H-6), 7.14 (1H; d; $J$ = 2.0 Hz; H-8), 5.38 (1H; d; $J$ = 7.5, H-1″), 4.27 (1H; d; $J$ = 7.5 Hz; H-1‴), 4.02 (6H, s, $OCH_3$-3′ and $OCH_3$-5′). $^{13}$C NMR (125 MHz, $CD_3OD$:$CF_3COOD$ (19:1)): $\delta$ 170.1 (C-7), 165.0 (C-2), 156.9 (C-5 and C-9), 148.2 (C-3′ and C-5′), 148.0 (C-4′), 126.4 (C-4), 113.7 (C-10), 111.2 (C-1′), 103.6 (C-1‴), 100.7 (C-2′ and C-6′), 96.1 (C-6), 94.3 (C-1″), 87.7 (C-8), 47.2 ($OCH_3$-3′ and $OCH_3$-5′).

### 2.4. Chromatographic analysis of fruits

Analytical TLC analyses were performed on reversed phase silica gel (RP-18) plates (Sorbent, Georgia, USA). Spots on TLC plates were visualized under UV light and by spraying anisaldehyde-$H_2SO_4$ followed by heating at 120 °C.

HPLC-PDA analysis was carried out on a gradient elution using solvent A (0.1% TFA in water) and solvent B (MeOH containing 0.1% TFA) in a gradient mode: 0 min, 5% B; 0–14 min, from 5% B to 35% B, with a dwell time of up to 25 min; 25–30 min, from 35% B to 100% B, with a dwell time of up to 35 min. An analytical column, Phenomenex C18-Hydro (250 × 4.6 mm, i.d.), was used with the following parameters: 4 μm, flow rate 1 mL·min$^{-1}$, room temperature, and injection volume was 10 μL. The UV spectra were recorded in the range 220–600 nm, and anthocyanins were detected at 520 nm. HPLC-PDA-MS/MS analyses were performed with the mass spectrometer in full scan mode. The following settings were applied throughout the analyses: capillary voltage 4.500 V; dry gas temperature 150 °C; dry gas flow 4 L min$^{-1}$; nebulizer gas nitrogen. A split of 1:3 of the LC eluent was used to introduce the sample into the stainless steel capillary probe, where the compounds were ionized. The chromatographic conditions were performed as mentioned above for HPLC-PDA. HPLC-PDA and HPLC-PDA-MS/MS along with comparison with literature data (Li et al., 2009; Reynertson et al., 2008) allowed the identification of eight anthocyanins.

### 2.5. Cell cultures

The HepG2 cell line was cultured in DMEM culture medium (Dulbecco's Modified Eagle Medium, Sigma-Aldrich Chemical Co., St. Louis, MO, USA), and the Hepa 1c1c7 cell line was cultured in α-MEM (Alpha Minimum Essential Medium Eagle, Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Both media were supplemented with 100 U·mL$^{-1}$ penicillin and streptomycin, 0.1 mg·mL$^{-1}$ sodium bicarbonate, kanamycin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), and a pH 7.2 and supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), and incubated at 37 °C in a humid atmosphere containing 5% $CO_2$. Cell suspensions at $8.0 \times 10^3$ and $1.0 \times 10^3$ cells per well densities were seeded in 96-well plates for Sulforhodamine B and Quinone reductase induction assays; and $2.5 \times 10^5$ cells per well and $1.0 \times 10^6$ cells per plate were seeded in 24-well plates and in 100 × 20 mm plastic dishes for the comet assay and micronucleus test, respectively.

### 2.6. Cell treatments

Cells were treated with the crude extracts and fractions of *E. jambolana* at concentration from 0.15 to 40 μg·mL$^{-1}$. This concentration range was chosen because there were no preliminary studies on the action of *E. jambolana*, it was performed a screening for the concentrations that presented genotoxic potential, it was decided to test lower concentrations, in order to delimit non-genotoxic concentrations to continue the other tests. The pre and post-treatments employed in the antigenotoxicity and antimutagenicity experiments were directed getting as a base the model described by Scolastici et al. (2008) for pre and simultaneous treatments.

The post-treatment was utilized the same time in order to compare the 24 h of pre-treatment. Moreover if 24 h presented spontaneous repair process the negative control will have the same result. We could verify a low comet score in the treatment when compared with negative control. Culture medium was used as a negative control and 0.1 M hydrogen peroxide ($H_2O_2$) for 5 min was used as a positive control. Treatments were performed in duplicate for 24 h and three independent experiments were carried out for each test.

### 2.7. Cytotoxicity assay by Sulforhodamine B (SRB)

SRB is an anionic dye that binds to basic amino acid residues, and thus stains cell proteins, allowing for spectrophotometric quantitation (at 570 nm). The determination of cytotoxicity was performed according to Skehan et al. (1990) and Voigt (2005). Briefly, cells HepG2 line, in a suspension of $1.5 \times 10^4$ cells/well, after 24 h of cultivation, they were treated with extracts and fractions of *E. jambolana* for 24 h, then cold trichloroacetic acid (TCA) was added, and there was incubation for 1 h at 4 °C. The TCA solution was removed and the plates were washed, it was added 50 μL of the 0.4% SRB solution (diluted in acetic acid) for 20 min. The SRB was removed, the plates were washed 3–4 times with 1% acetic acid, and the dye dissolved with 10 mM Tris Base (Sigma). The reading was carried out at 570 nm. The tests were performed in three independent experiments and the percentage of live cells was calculated in relation to the negative control, representing the cytotoxicity of each treatment, as proposed by Zhang et al., 2004: Live cells (%) = Test Absorbance × 100/Negative Control Absorbance.

### 2.8. Comet assay

The comet assay was performed according to a protocol established by Singh, McCoy, Tice, and Schneider (1988). The cell suspension $2.5 \times 10^5$ cells/well was grown in 24 wells, after 24 h of culture, the cells were treated with extracts and fractions of *E. jambolana*. For 24 h, subsequently the treatments were removed, the cells were washed, trypsinized, reserved and centrifuged at 1500 rpm for 3 min. After centrifugation, the cells were resuspended in 200 μL of LMP agarose at 37 °C, next transferred to 2 slides, previously treated with normal melting point agarose. The slides were recovered with coverslip for 5 min at 8 °C protected from light. The coverslips were removed and the slides were subjected to the freshly prepared lysis solution at 4 °C for 12 h under refrigeration and protected from light. Then the slides were submitted to electrophoretic running. Then the foils were neutralized at 4 °C for 15 min, fixed in 100% ethanol and stained with ethidium bromide solution. The reading was performed under a fluorescence microscope with 516–560 nm filter. For each test sample and their respective concentrations, 50 cells were analyzed by the software TriTek CometScore™ version 1.5. The percentage of DNA in the tail was used for the analysis of fragmented DNA amounts (Moller, 2005). The experimental design for evaluation of genotoxicity of the tested samples is showed in Fig. 1A.

#### 2.8.1. Evaluation of antigenotoxicity

In order to evaluate the antigenotoxicity of extracts and fractions was used as damage inducing agent the hydrogen peroxide ($H_2O_2$) in HepG2 cells. Pre-treatment and post-treatment protocols were directed getting as a base the model described by Scolastici et al. (2008).

In the pre-treatment the cells were submitted to different non-genotoxic concentrations of extracts and fractions, after 24 h the culture medium with the treatments were removed and was added 500 μL of the damage inducer, $H_2O_2$ diluted in culture medium at 0.1 M for 5 min. Then, the cells were washed, trypsinized, centrifuged and the protocol was performed as described above. In the post-treatment, the cells were first submitted to the damage inducer, $H_2O_2$ diluted in culture medium, at 0.1 M for 5 min, then the $H_2O_2$ was removed and the cells were exposed to different non-genotoxic concentrations of the treatments. After 24 h the medium was removed, the cells were washed, trypsinized, centrifuged and the protocol was performed as previously described. The experimental design for evaluation of antigenotoxicity with pre-treatment and post-treatment of the tested samples is showed in Fig. 1B and C, respectively.

### 2.9. Micronucleus assay

The micronucleus assay with cytokinesis block using cytochalasin B was performed as described by Fenech (2000). HepG2 cells were grown and after 24 h of culture, the cells were treated with extracts and fractions of *E. jambolana*. After 24 h the culture medium with the treatments was removed, and a new culture medium containing cytochalasin B at the concentration of 2.4 μg/mL was added, for 48 h and protected from light. After 48 h, the cells were washed, trypsinized and centrifuged at 1200 rpm for 3 min. The supernatant was removed and the cells resuspended in cold KCl solution and fixative (methanol and acetic acid solution), centrifuged at 800 rpm for 8 min. The supernatant was discarded and 4 mL of the methanol and acetic acid solution was added, with two drops of formaldehyde, the cells were centrifuged at 800 rpm for 6 min. Then, of the supernatant was removed, the cells were resuspended and spread over several slides. Cells were stained with Giemsa solution, and 1000 binucleated cells with intact cytoplasm and clearly delimited nuclei were scored and analyzed for each test, based on the frequency of micronuclei and nuclear division index. The frequency of micronuclei and IDNs were calculated using three independent experiments. The IDN was given by the formula IDN = [M1 + 2 (M2) + 3 (M3) + 4 (M4)]/N, where M1 to M4 represent the number of cells with 1, 2, 3 and 4 nuclei and N Represents the total number of cells, according to Eastmond and Tucker (1989). The frequency of micronuclei and IDNs were calculated from three independent experiments. The experimental design for evaluation of mutagenicity of the tested samples is showed in Fig. 1D.

#### 2.9.1. Evaluation of antimutagenicity

In the pre-treatment the cells were submitted to different non-genotoxic concentrations of extracts and fractions of *E. jambolana*, after 24 h the culture medium with the treatments were removed and was added 5 ml of the damage inducer, 0.1 M $H_2O_2$, diluted in culture medium, for 5 min. Then the $H_2O_2$ was removed and the culture medium containing cytochalasin B (to stop cytokinesis) at the concentration of 2.4 μg/ml was added, for 48 h and protected from light. In the post-treatment the cells were first subjected to the damage inducer, 0.1 M $H_2O_2$ diluted in culture medium, for 5 min, then the $H_2O_2$ was removed and the cells were exposed to different non-genotoxic concentrations of the treatments, and it followed the Fenech (2000) protocols. The experimental design for evaluation of antimutagenicity with pre-treatment and post-treatment of the tested samples is showed in Fig. 1E and F, respectively.

No spontaneous DNA repair was observed in antigenotoxicity and antimutagenicity experiments during standard experiments (data nor showed).

### 2.10. Quinone reductase induction assay

The induction and activity of quinone reductase enzyme was assayed using Hepa 1c1c7 cells. The treatments were carried out

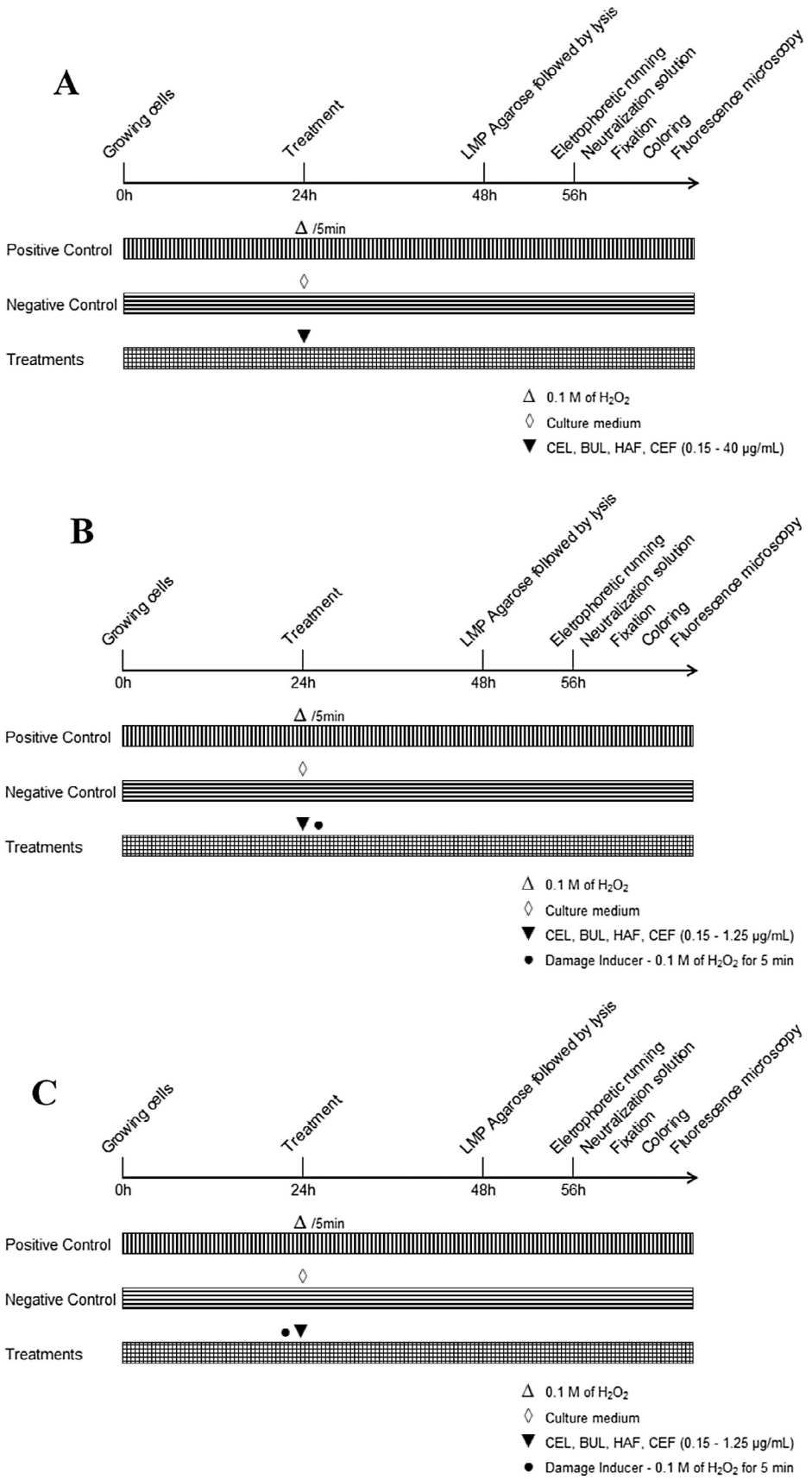



**Fig. 1.** Experimental design of the crude extract leaves (CEL), crude extract fruits (CEF), *n*-buthanolic fraction leaves (BuL) and hydroalcoholic fraction fruits (HAF) of *E. jambolana* for the evaluation of genotoxicity (A), antigenotoxicity with pre-treatment (B), antigenotoxicity with post (C), mutagenicity (D), antimutagenicity with pre-treatment (E), antimutagenicity with post treatment (F).

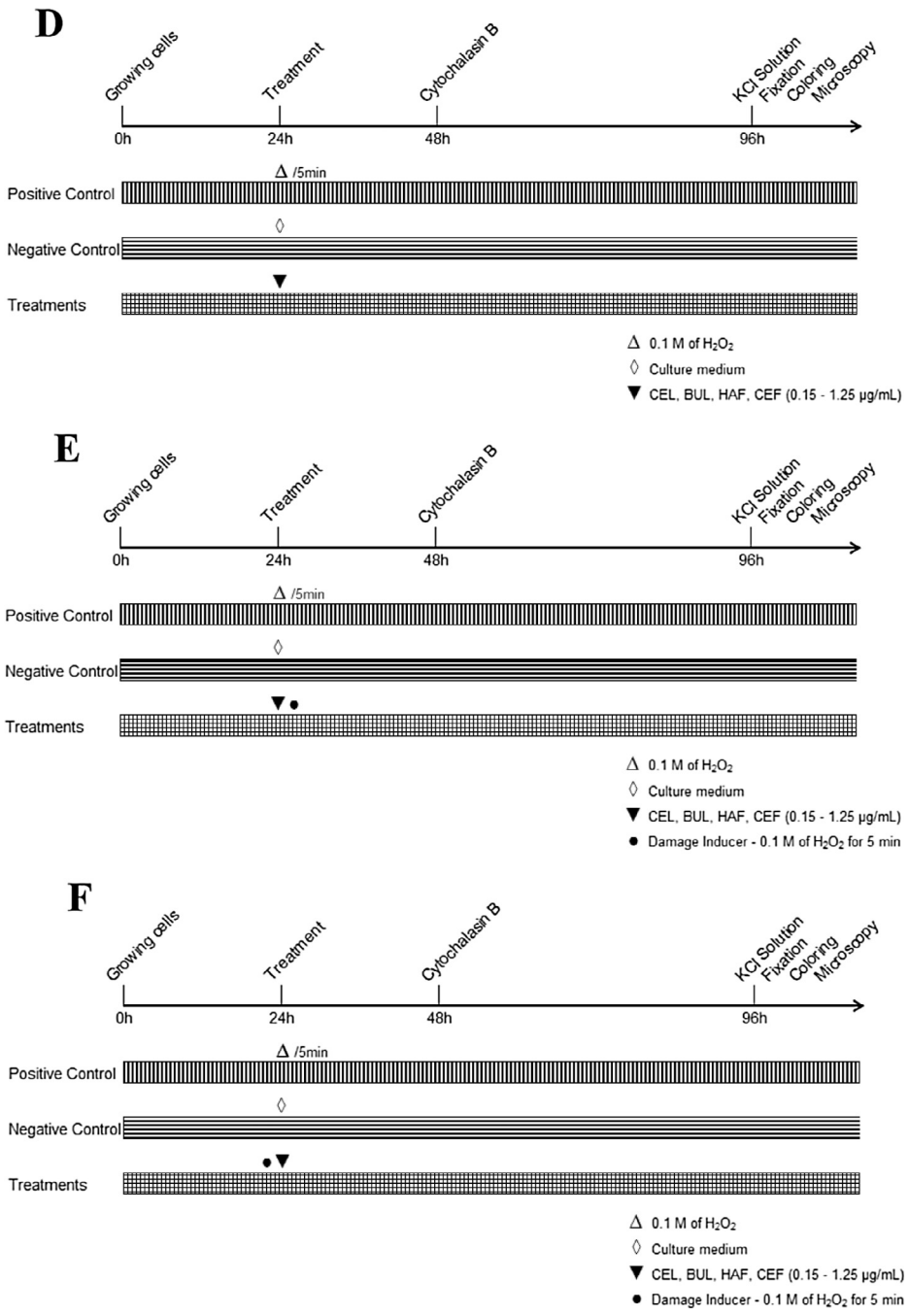



**Fig. 1** (*continued*)

for 48 h to evaluate the enzymatic activity as described by Kang and Pezzuto (2004). In this test, a direct measurement of enzyme activity is obtained as the quinone reductase catalyzes the reduction of menadiol to menadione, which promotes a non-enzymatic reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazo lium (MTT). Such reduction involves NADPH as an electron donor, and leads to formazan (reduced MTT), producing a blue color, which is detected by spectrophotometry.

### *2.11. Statistical analyses*

The quantitative results of Genotoxicity and antigenotoxicity of *E. jambolana* treatment compared to the negative control and positive control, respectively, was applied the Kruskal Wallis test with Dunn post-test. Analyzes were performed with GraphPad Prism® software Version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). For frequency of micronuclei compared to the negative and positive control (mutagenicity and antimutagenicity, respectively) was used One-way variance analysis (ANOVA) with Dunnett post-test and for IDNs the applied test was One-way variance analysis (ANOVA) with Tukey post-test, with the aid of software BioEstat 5.0. For the application of One-way variance analysis (ANOVA) were firstly evaluated if the data had normal distribution. In all statistical tests were considered the significance level of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

## 3. Results and discussion

In our research, the crude extract and its fractions from fruits and leaves of *E. jambolana* were investigated regarding their

genotoxicity, antigenotoxicity, mutagenicity and antimutagenicity using a human hepatoma cell line (HepG2). Chromatographic purifications of BuL afforded two flavonoids, tricetin-4′-*O*-α-L-rhamnopyranoside (**1**) (Nazemiyeh et al., 2008) and myricitrin (**2**) (Mahmoud et al., 2001) (Fig. 2), which were characterized by analysis of $^{1}$H- and $^{13}$C NMR spectral data and comparison with published spectral data of similar compounds.

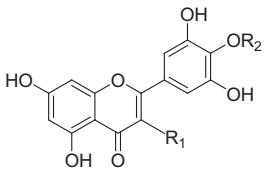
Chromatographic purifications of HAF afforded one pure anthocyanin, malvidin-3-*O*-gentiobiosyl (Fig. 2), which was characterized by NMR experiments ($^{1}$H, HMBC, and HMQC) and MS analysis. The molecular formula $C_{29}H_{35}O_{17}$ was determined by HRESIMS ions at *m*/*z* 655.1860 (malvidin-3-*O*-gentiobioside, $[M]^+$, calcd 655.1874).

The $^{1}$H-NMR spectrum of compound **3** exhibited signals for four aromatic protons, including two doublets at $\delta_H$ 7.09 (d, *J* = 2.0, H-6) and 7.14 (d, *J* = 2.0, H-8) assigned to protons at *meta* positions on ring A, and two singlets at $\delta_H$ 9.17 (s, H-4) and $\delta_H$ 8.04 (s, H-2′and H-6′), two signals for methoxyls [$\delta_H$ 4.02 (s, $OCH_3$-3′ and $OCH_3$-5′)], and two signals associated with anomeric protons at $\delta_H$ 5.38 (d, *J* = 7.5 H-1″) and $\delta_H$ 4.27 (d, *J* = 7.5 Hz, H-1‴) (Shoyama et al., 1990). The HMBC experiment allowed the assignment of signals for fifteen aromatic carbons [$\delta_C$ 170.1 (C-7), 165.0 (C-2), 156.9 (C-5 and C-9), 147.0 (C-3), 148.2 (C3′ and C-5′), 148.0 (C-4′), 126.4 (C-4), 113.7 (C-10), 111.2 (C-1′), 100.7 (C-2′ and C-6′), 96.1 (C-6), and 87.7 (C-8)], two methoxy groups [$\delta_C$ 47.2 ($OCH_3$-3′ and $OCH_3$-5′)], and two anomeric carbons [$\delta_C$ 103.6 (C-1‴) and 94.3 (C-1″)]. The HMBC experiment also showed interactions of H-1″ with C-3, and H-6 with C-1‴ evidencing the interglucosyl bond 1 → 6. In addition, the anthocyanin was analyzed by MS/MS, producing $[M]^+$ ions at *m*/*z* 655, and its fragments at *m*/*z* 493 and 331 corresponding to malvidin-3-*O*-glucosyl and the aglycone malvidin, respectively. The literature shows characteristic fragmentation patterns for anthocyanin diglycosides, which contributes to their structural determination (Wu & Prior, 2005a, 2005b; Giusti, Rodriguez-Saona, Griffin, & Wrolstad, 1999). Spectra of 3,5-diglycosyl anthocyanins may display fragments corresponding to the aglycone, to the C3-glycosyl-anthocyanin and C5-glycosyl-anthocyanin moieties, in addition to the molecular parent ion (Giusti et al., 1999). On the other hand, C-3-diglycosyl anthocyanins, bearing a sophoroside, sambubioside or laminaribioside moiety, have been shown to exhibit only one fragment from the MS/MS experiment, suggesting absence of the interglucosyl bond cleavage (Giusti et al., 1999). Exceptions to this pattern have been found for C-3-rutinosyl-anthocyanins, which led to the formation of additional fragments corresponding to a monoglucosyl-anthocyanin, resulting from the interglucosyl bond cleavage due to the enhanced rotation of the 1 → 6 linkage between the rhamnopyranosyl and glucopyranosyl moieties, and the easier accessibility of the gas used to produce fragmentation (Wu & Prior, 2005a, 2005b). Gentiobioside is a diglucoside bearing similarities to rutinoside, as the interglucosyl linkage (1 → 6) between two glucosyl moieties, which might be associated to the easier interglucosyl bond fragmentation. On the other hand, sophoroside (glucose 1 → 2 glucose), sambubioside (xylose 1 → 2 glucose) and laminaribioside (glucose 1 → 3 glucose), which bear more stable sugar bonds, did not have their interglucosyl linkage cleaved in tandem experiments (Giusti et al., 1999). MS data corroborated those from NMR spectra, and compound **3** was identified as the anthocyanin malvidin-3-*O*-gentiobioside.

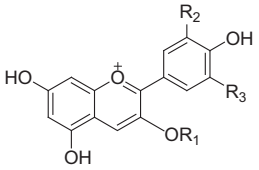
Analysis of CEF by HPLC-PDA-MS/MS evidenced the presence of additional anthocyanins from the observation of mass fragments at *m*/*z* 303, 287, 317, and 331, corresponding to delphinidin, cyanidin, petunidin and malvidin aglycones, respectively. Fig. 3 shows the HPLC-PDA chromatogram of CEF, and peaks of anthocyanins tentatively identified by HPLC-PDA-MS/MS in the positive mode are described in Table 1. The identification of anthocyanins diglucosides (peaks 1–4) were carried out based on fragmentation patterns (Wu & Prior, 2005b; Giusti et al., 1999) and from NMR analyses obtained from compound **3**.

Peaks 1 to 4, with molecular ions at *m*/*z* 627, 611, 641, and 655, correspond to anthocyanins diglucosides tentatively identified as delphinidin-3-*O*-gentiobioside, cyanidin-3-*O*-gentiobioside, petunidin-3-*O*-gentiobioside, and malvidin-3-*O*-gentiobioside, respectively. Tandem mass spectrometry (MS/MS) evidenced the same fragmentation pattern with cleavage of the interglucosyl bond of gentiobioside, leading to fragments corresponding to the aglycone, to the C-3-monoglucosyl-anthocyanin moiety, and the molecular parent ions for all anthocyanins diglucosides. Delphinidin-3-*O*-gentiobiside was tentatively identified from the ion at *m*/*z* 627 and mass fragments at *m*/*z* 465 and 303, corresponding to the delphinidin-3-*O*-glucoside moiety and the aglycone delphinidin, respectively. Cyanidin-3-*O*-gentiobiside was tentatively identified from the ion at *m*/*z* 611 and mass fragments at *m*/*z* 449 and 287, corresponding to the delphinidin-3-*O*-glucoside moiety and the aglycone delphinidin, respectively. Petunidin-3-*O*-gentiobioside was tentatively identified from the ion at *m*/*z* 641 and mass fragments at *m*/*z* 479 and 317, assigned to the petunidin-3-*O*-glucoside moiety, and the aglycone petunidin, respectively.

Although NMR experiments were only performed for one anthocyanin, tandem MS data allowed the identification of three additional anthocyanin diglucosides. Anthocyanins may be classified as sugar-dependent or aglycone-dependent (Wu & Prior, 2005b) Aglycone-dependent anthocyanins bear the same aglycone and different sugars. Conversely, sugar-dependent anthocyanins bear different aglycones and the same sugar moiety, which is the




**1** $R_1$ = H, $R_2$ = rhamnose
**2** $R_1$ = O-rhamnose, $R_2$ = H




**3** $R_1$ = gentibioside, $R_2$ = $OCH_3$, $R_3$ = $OCH_3$
**4** $R_1$ = gentibioside, $R_2$ = OH, $R_3$ = OH
**5** $R_1$ = gentibioside, $R_2$ = OH, $R_3$ = $OCH_3$
**6** $R_1$ = gentibioside, $R_2$ = H, $R_3$ = OH
**7** $R_1$ = glucoside, $R_2$ = OH, $R_3$ = OH
**8** $R_1$ = glucoside, $R_2$ = H, $R_3$ = OH
**9** $R_1$ = glucoside, $R_2$ = OH, $R_3$ = $OCH_3$
**10** $R_1$ = glucoside, $R_2$ = $OCH_3$, $R_3$ = $OCH_3$

**Fig. 2.** Structures of compounds: Tricetin-4′-*O*-α-L-rhamnopyranoside (**1**); Myricitrin (**2**); Malvidin-3-*O*-gentibioside (**3**); Delphinidin-3-*O*-gentibioside (**4**); Cyanidin-3-*O*-gentibioside (**5**); Petunidin-3-*O*-gentibioside (**6**); Delphinidin-3-*O*-glucoside (**7**); Cyanidin-3-*O*-glucoside (**8**); Petunidin-3-*O*-glucoside (**9**); Malvidin-3-*O*-glucoside (**10**).

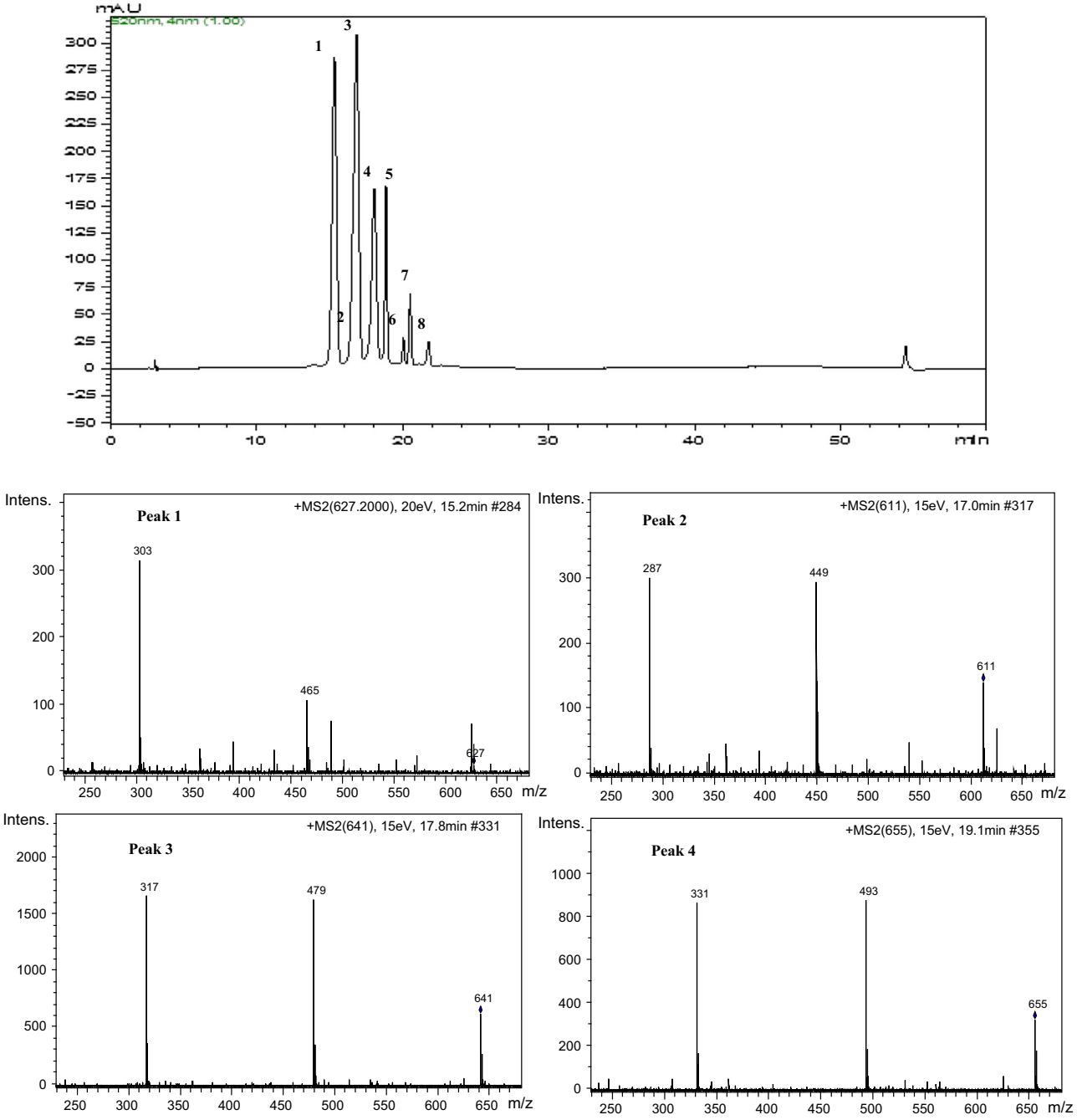



**Fig. 3.** Reverse-phase HPLC-PDA chromatogram of anthocyanin profile of *E. jambolana* extract and MS/MS spectra of peak 1, 2, 3, and 4 of Delphinidin-3-*O*-gentibioside (**4**), Cyanidin-3-*O*-gentibioside (**5**), Petunidin-3-*O*-gentibioside (**6**) and malvidin-3-*O*-gentibioside (**3**), respectively. Refer to Table 1 for the identification of each peak.

**Table 1**
Anthocyanins tentatively identified by HPLC-PDA-MS/MS in CEF.

| Peak | $R_T$ (min) | $[M]^+$ | MS/MS- | Anthocyanins |
|---|---|---|---|---|
| 1 | 15.9 | 627 | 465/303 | Delphinidin-3-*O*-gentiobioside (**4**) |
| 2 | 16.7 | 611 | 449/287 | Cyanidin-3-*O*-gentiobioside (**6**) |
| 3 | 17.6 | 641 | 479/317 | Petunidin-3-*O*-gentiobioside (**5**) |
| 4 | 19.7 | 655 | 493/331 | Malvidin-3-*O*-gentiobioside (**3**) |
| 5 | 22.1 | 465 | 303 | Delphinidin-3-*O*-glucoside (**7**) |
| 6 | 25.9 | 449 | 287 | Cyanidin-3-*O*-glucoside (**8**) |
| 7 | 28.4 | 479 | 317 | Petunidin-3-*O*-glucoside (**9**) |
| 8 | 29.3 | 493 | 331 | Malvidin-3-*O*-glucoside (**10**) |

case for anthocyanins diglucosides tentatively identified from *E. jambolana* (Wu & Prior, 2005a, 2005b). In addition to anthocyanin diglucosides, peaks 5 to 8 with molecular ions at *m*/*z* 465, 449, 479, and 493, corresponding to anthocyanin monoglucosides, generated fragments in tandem experiments at *m*/*z* 303, 287, 317 and 331 for their aglycones, and allowed their tentatively identification as delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside, respectively (Li et al., 2009; Reynertson et al., 2008).

### 3.1. Cytotoxicity evaluation

The extracts and fractions showed a similar profile of cell viability, with 20–40% cell death for most samples, even at higher concentrations. However, the extracts and fractions from fruits (CEF and HAF) showed a higher percentage of living cells (Fig. 4).

Cancer preventive strategies are considered promising, especially when associated with natural products present in widely used food or medicinal plants. In order to be considered as useful chemopreventive agents, compounds, semi-purified fractions or crude extracts should exhibit low cytotoxicity besides their chemopreventive activity (Yang & Liu, 2009). Therefore, the extracts and fractions from *E. jambolana* leaves and fruits were evaluated for their genotoxicity and mutagenicity, in addition to chemopreventive potential.

### 3.2. Assessment of genotoxicity and mutagenicity

Several natural products, which are widely used as folk medicines, can be potentially genotoxic and mutagenic. Such profiles must be evaluated before the products can be determined as useful chemopreventive agents.

Genotoxic and mutagenic effects of *E. jambolana* samples are shown in Fig. 5 (A) and Table 2, respectively. CEL demonstrated genotoxic effect at 5 $\mu g \cdot mL^{-1}$ (13.3 ± 1.2%) ($p < 0.05$) and 10 $\mu g \cdot mL^{-1}$ (14.1 ± 1.3%) ($p < 0.001$), which was statistically different from the negative control. Genotoxic effects at other concentrations were not significantly different when compared to the negative control. BuL also presented genotoxic potential at 5 $\mu g \cdot mL^{-1}$ (13.4 ± 1.3%) and 10 $\mu g \cdot mL^{-1}$ (16.3 ± 1.2%), which were both statistically different from the negative control ($p < 0.01$ and $p < 0.001$, respectively). As observed for CEL, genotoxic effects of BuL at other test concentrations were not significantly different when compared to the negative control and therefore were not considered genotoxic.

Both samples from fruits, CEF and HAF, exhibited genotoxic profiles similar to the samples obtained from the leaves at the highest concentrations. CEF was genotoxic between 5 $\mu g \cdot mL^{-1}$ (11.8 ± 1.1%) and 10 $\mu g \cdot mL^{-1}$ (13.5 ± 1.4%), with $p < 0.01$ and $p < 0.001$, respectively, and HAF (Table 2) was also genotoxic at 5 $\mu g \cdot mL^{-1}$ (12.3 ± 1.2%) and 10 $\mu g \cdot mL^{-1}$ (13.1 ± 1.6%) (both with $p < 0.001$). No statistically significant differences were observed at lower concentrations when compared to the negative control.

The treatment of HepG2 cell line with the positive control ($H_2O_2$ 0.1 M) showed a significant increase in FMN (frequency of micronuclei) when compared with the negative control (cells treated with culture media). Similar results were observed after analysis of NDI (nuclear cell division index), indicating a lack of uniformity in the nuclear division of the positive control.

No significant differences in FMN and NDI were observed for CEL, whereas BuL showed increase in FMN for all concentrations tested when compared with the negative control, and NDI did not present a statistical difference when compared to the negative control, suggesting uniformity in nuclear division.

Significant increases in FMN were not observed for CEF and HAF at the tested concentrations, and therefore no mutagenic effects were detected. When evaluating NDI, both extracts and fractions exhibited uniformity during the nuclear division at all concentrations tested.

Mutagenicity by micronucleus assay in HepG2 cell line, after 24 h of treatment with crude extract (CEL) and *n*-butanol fraction (BuL) of leaves, crude extract (CEF) and hydro-alcoholic fraction (HAF) of fruits from *E. jambolana*; culture media was used as negative control (NC), and $H_2O_2$ at 0.1 M as positive control. Results are demonstrated as mean of FMN: Frequencies of Micronuclei in 1000 binucleated cells ± standard error (SE), and mean of NDI: Nuclear cell Division Index ± standard error (SE). Three independent experiments were analyzed by One-way ANOVA with Dunnett's post-test for FMN and Tukey's post-test for NDI. The results of samples were compared with negative control (Statistical differences: $*$ indicates $P < 0.05$; $**$ indicates $P < 0.01$ and $***$ indicates $P < 0.001$). Statistical analyses were performed with software Graphpad Prism 5.0.

The comet assay is known to detect primary lesions to DNA, or repairable damage, whereas the micronucleus test detects irreparable damage (Salvadori et al., 1993; Scolastici et al., 2008). Therefore, it may be suggested that the current results indicate that only BuL was able to cause irreparable damage to DNA at lower concentrations, as observed in the micronucleus test. On the other hand, the comet assay and lower concentrations (2.5 $\mu g \cdot mL^{-1}$) for the extracts and fractions from *E. jambolana* did not cause DNA damage.

### 3.3. Assessment of antigenotoxicity and antimutagenicity

The occurrence rate of cancer and other diseases caused by genotoxic agents are continually increasing worldwide and the determination of antigenotoxic and antimutagenic activities of

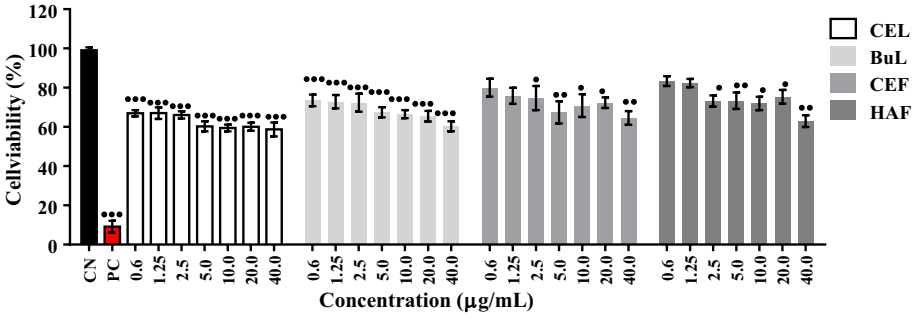



**Fig. 4.** Cytotoxicity by Sulforhodamine B assay in HepG2 cell line, after 24 h of treatment with crude extract (CEL), *n*-butanol fraction (BuL) of leaves, crude extract (CEF), hydroalcoholic fraction (HAF) of fruits from *E. jambolana*, culture media as Negative control (NC), and $H_2O_2$ at 0.1 M as Positive control (PC). Results are demonstrated as mean of Percentage of DNA ± standard error (SE) of three independent experiments analyzed by On-way ANOVA test with Tukey post-test compared with negative control (Statistical differences: • indicates $P < 0.05$; •• indicates $P < 0.01$ and ••• indicates $P < 0.001$). Statistical analyses were performed with software Graphpad Prism 5.0.

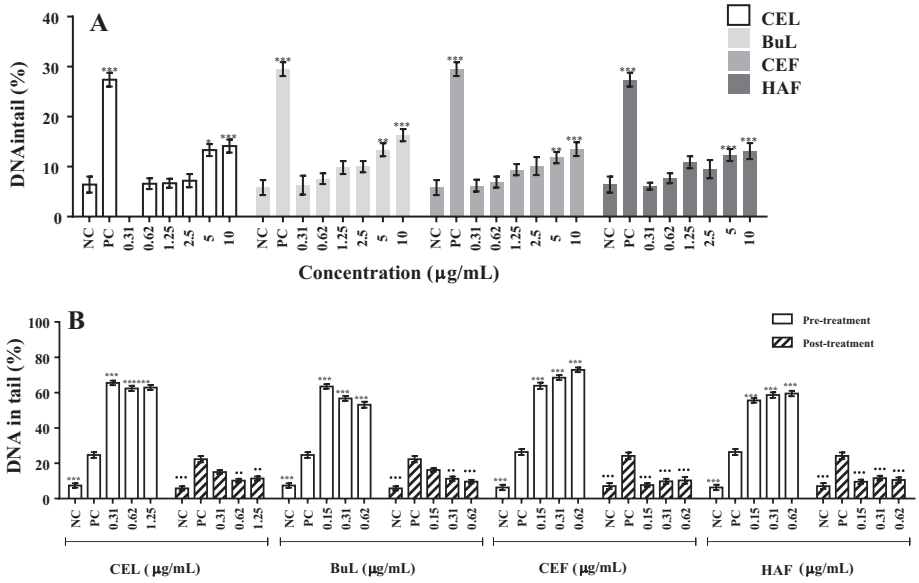



**Fig. 5.** Genotoxicity (A) and Antigenotoxicity (B) by Comet assay in HepG2 cell line, after treatment with crude extract (CEL), *n*-butanol fraction (BuL) of leaves, crude extract (CEF), hydroalcoholic fraction (HAF) of fruits from *E. jambolana*, culture media as Negative control (NC), and $H_2O_2$ at 0.1 M as Positive control (PC). Results are demonstrated as mean of Percentage of DNA ± standard error (SE) of three independent experiments analyzed by Kruskal-Wallis test with Dunn's post-test compared with negative control (Statistical differences for genotoxicity and antigenotoxicity pre-treatment assay: * indicates $P < 0.05$; ** indicates $P < 0.01$ and *** indicates $P < 0.001$; Statistical differences for post-treatment assay: • indicates $P < 0.05$; •• indicates $P < 0.01$ and ••• indicates $P < 0.001$). Statistical analyses were performed using the software Graphpad Prism 5.0.

**Table 2**
Mutagenicity of crude extract (CEL) and *n*-butanol fraction (BuL) from leaves and crude extract (CEF) and hydro-alcoholic fraction (HAF) from fruits from *E. jambolana*.

| | Concentration (µg·mL$^{-1}$) | FMN | NDI |
|---|---|---|---|
| CEL | $H_2O_2$ (0.1 M) | 155.7 ± 5.8*** | 1.4 ± 0.06*** |
| | NC | 26.3 ± 4.1 | 1.6 ± 0.1 |
| | 0.31 | 40.0 ± 4.2 | 1.5 ± 0.1 |
| | 0.62 | 39.0 ± 3.1 | 1.6 ± 0.1 |
| | 1.25 | 43.7 ± 3.2 | 1.6 ± 0.1 |
| BuL | $H_2O_2$ (0.1 M) | 155.7 ± 5.8*** | 1.4 ± 0.1*** |
| | NC | 26.3 ± 4.1 | 1.6 ± 0.1 |
| | 0.15 | 48.3 ± 2.6** | 1.5 ± 0.1 |
| | 0.31 | 48.0 ± 3.1** | 1.6 ± 0.1 |
| | 0.62 | 46 ± 1.6** | 1.5 ± 0.1 |
| CEF | $H_2O_2$ (0.1 M) | 155.7 ± 5.8*** | 1.4 ± 0.1*** |
| | NC | 26.3 ± 4.1 | 1.6 ± 0.1 |
| | 0.15 | 31.7 ± 3.2 | 1.5 ± 0.1 |
| | 0.31 | 26.7 ± 2.3 | 1.5 ± 0.1 |
| | 0.62 | 29.0 ± 2.1 | 1.5 ± 0.1 |
| HAF | $H_2O_2$ (0.1 M) | 155.7 ± 5.8*** | 1.4 ± 0.1*** |
| | NC | 26.3 ± 4.1 | 1.6 ± 0.1 |
| | 0.15 | 24.0 ± 3.2 | 1.5 ± 0.1 |
| | 0.31 | 26.3 ± 2.6 | 1.5 ± 0.1 |
| | 0.62 | 34.0 ± 4.6 | 1.5 ± 0.1 |

plant extracts is important in the discovery of new and effective treatments using natural products. Within this context, two experimental designs (pre-and post-treatment) were carried out using $H_2O_2$, to understand the mechanisms of antimutagenicity and antigenotoxicity of *E. jambolana* extracts on HepG2 cells. The results of antigenotoxicity and antimutagenicity are demonstrated in Fig. 5 (B) and Table 3, respectively.

CEL was evaluated for its antigenotoxic profile at concentrations of 0.31, 0.62 and 1.25 µg·mL$^{-1}$. Pre-treatment with this extract triggered an increase in DNA damage by the mutagen ($H_2O_2$) of 65.5 ± 1.3, 62.4 ± 1.5, 62.9 ± 1.4% at the tested concentrations, respectively, showing a statistically significant difference when compared to the positive control $p < 0.001$, exceeding the result obtained with the positive control (24.7 ± 1.6%).

In the pre-treatment with BuL, damage to cellular DNA was observed, with concentrations of 0.15, 0.31 and 0.62 µg·mL$^{-1}$ leading to 63. 5 ± 1.3, 56.7 ± 1.4, 53.1 ± 1.7% of DNA fragmentation, respectively, which evidenced statistical differences when compared to the positive control ($p < 0.001$). The post-treatment with BuL demonstrated a reduction in DNA damage at concentrations of 0.31 (11.2 ± 1.3%) and 0.62 µg·mL$^{-1}$ (9.6 ± 1.0%), showing a statistical difference when compared to the positive control ($p < 0.01$ and $p < 0.001$), respectively. However, the concentration of 0.15 µg·mL$^{-1}$ did not present a statistical difference in relation to the positive control, and was not considered antigenotoxic.

CEF and HAF were also tested at concentrations of 0.15, 0.31 and 0.62 µg·mL$^{-1}$. At pre-treatment conditions, CEF presented 63.9 ± 1.8, 68.5 ± 1.4 and 72.9 ± 1.3% of DNA in the tail (fragmented DNA), with statistical differences when compared to the positive control, $p < 0.001$. The same concentrations were used for HAF, 0.15, 0.31 and 0.62 µg·mL$^{-1}$, which led to 55.6 ± 1.3, 58.6 ± 1.6 and 59.5 ± 1.5% of DNA damage, respectively, with a statistical significance ($p < 0.001$) when compared to the positive control.

At post-treatment conditions, the three concentrations of CEF and HAF led to statistical differences when compared to the positive control, demonstrating the ability to decrease the damage caused by $H_2O_2$ after a recovery time. CEF showed 7.7 ± 1.1 (0.15 µg·mL$^{-1}$), 9.8 ± 1.4 (0.31 µg·mL$^{-1}$) and 10.3 ± 1.9% (0.62 µg·mL$^{-1}$) of DNA in the tail, with $p < 0.001$, whereas HAF showed 9.5 ± 1.2 (0.15 µg·mL$^{-1}$), 11.6 ± 1.3 (0.31 µg·mL$^{-1}$) and 10.5 ± 1.5% (0.62 µg·mL$^{-1}$) of fragmented DNA, with $p < 0.001$.

According to the results of the present study, pre-treatment with *E. jambolana* did not protect HepG2 cells against genotoxic effects of $H_2O_2$ (even boosting the damage), although at post-treatment conditions, antigenotoxic action was observed. Extracts and fractions from *E. jambolana* were shown to act in the cancer cells used in this study after induction of DNA damage, probably exerting a stimulatory effect on the DNA repair system.

In pre-treatment conditions with CEL, there was a significant reduction in FMN when compared with the positive control in all tested concentrations. The Nuclear Division Index (NDI) was also calculated to examine whether cell division occurred uniformly for all treatments. For CEL pre-treatment at the tested

**Table 3**
Antimutagenicity of crude extract (CEL) and *n*-butanol fraction (BuL) of leaves, and crude extract (CEF) and hydroalcoholic fraction (HAF) of fruits from *E. jambolana*.

| | Concentration ($\mu g \cdot mL^{-1}$) | Pre-treatment | | Post-treatment | |
|---|---|---|---|---|---|
| | | FMN | NDI | FMN | NDI |
| CEL | $H_2O_2$ (0.1 M) | 184.0 ± 10.4 | 1.6 ± 0.1 | 206.0 ± 4.9 | 1.7 ± 0.1 |
| | NC | 19.3 ± 2.6*** | 1.6 ± 0.1 | 24.0 ± 3.6*** | 1.7 ± 0.1 |
| | 0.31 | 98.6 ± 7.5*** | 1.5 ± 0.1 | 59.0 ± 6.4*** | 1.6 ± 0.1 |
| | 0.62 | 47.0 ± 8.3*** | 1.5 ± 0.1 | 61.6 ± 7.5*** | 1.4 ± 0.1** |
| | 1.25 | 42.7 ± 2.3*** | 1.5 ± 0.1 | 34.0 ± 4.3*** | 1.5 ± 0.1* |
| BuL | $H_2O_2$ (0.1 M) | 184.0 ± 10.4 | 1.6 ± 0.1 | 206.0 ± 4.9 | 1.7 ± 0.1 |
| | NC | 19.3 ± 2.6*** | 1.6 ± 0.1 | 24.0 ± 3.6*** | 1.7 ± 0.1 |
| | 0.15 | 57.6 ± 5.5*** | 1.3 ± 0.1*** | 81.3 ± 8.4*** | 1.6 ± 0.1 |
| | 0.31 | 34.3 ± 4.3*** | 1.3 ± 0.1*** | 71.0 ± 6.2*** | 1.5 ± 0.1* |
| | 0.62 | 24.0 ± 2.8*** | 1.5 ± 0.1 | 77.3 ± 5.2*** | 1.6 ± 0.1 |
| CEF | $H_2O_2$ (0.1 M) | 184.0 ± 10.4 | 1.6 ± 0.1 | 206.0 ± 4.9 | 1.7 ± 0.1 |
| | NC | 19.3 ± 2.6*** | 1.6 ± 0.1 | 24.0 ± 3.6*** | 1.7 ± 0.1 |
| | 0.15 | 53.6 ± 6.7*** | 1.6 ± 0.1 | 42.3 ± 3.7*** | 1.4 ± 0.1*** |
| | 0.31 | 45.0 ± 5.0*** | 1.6 ± 0.1 | 31.6 ± 4*** | 1.3 ± 0.1*** |
| | 0.62 | 47.3 ± 2.7*** | 1.6 ± 0.1 | 23.6 ± 3.1*** | 1.4 ± 0.1*** |
| HAF | $H_2O_2$ (0.1 M) | 184.0 ± 10.4 | 1.6 ± 0.1 | 206.0 ± 4.9 | 1.7 ± 0.1 |
| | NC | 19.3 ± 2.6*** | 1.6 ± 0.1 | 24.0 ± 3.6*** | 1.7 ± 0.1 |
| | 0.15 | 46.5 ± 7.3*** | 1.4 ± 0.1*** | 63.3 ± 3.4*** | 1.6 ± 0.1 |
| | 0.31 | 47.0 ± 6.4*** | 1.5 ± 0.1 | 46.6 ± 4.3*** | 1.6 ± 0.1 |
| | 0.62 | 44.6 ± 5.7*** | 1.6 ± 0.1 | 32.3 ± 3.1*** | 1.6 ± 0.1 |

Antimutagenicity by Micronucleus assay in HepG2 cell line, after 24 h of treatment with crude extract (CEL), *n*-butanol fraction (BuL) of leaves, crude extract (CEF), hydroalcoholic fraction (HAF) of fruits from *E. jambolana*, culture media as Negative control (NC), and $H_2O_2$ at 0.1 M as Positive control. Results are demonstrated as mean of FMN: Frequencies of Micronuclei in 1000 binucleated cells ± standard error (SE) and mean of NDI: Index of nuclear cell division ± standard error (SE). Three independent experiments were analyzed by One-way ANOVA with Dunnett's post-test for FMN and Tukey's post-test for NDI. The results of samples were compared with positive control. Statistical analyses were performed with software Graphpad Prism 5.0.

* Statistical difference indicates $P < 0.05$.
** Statistical difference indicates $P < 0.01$.
*** Statistical difference indicates $P < 0.001$.

concentrations, NDI did not show a statistically significant difference in relation to the negative control. A greater reduction in FMN after post-treatment with CEL was observed, when compared with the pre-treatment and the positive control at the concentrations tested, with statistical significance in relation to the positive control ($p < 0.001$). NDI showed statistical difference from the negative control only at concentrations of 0.62 and 1.25 $\mu g \cdot mL^{-1}$.

Pre-treatment with BuL demonstrated a significant reduction in the frequency of micronuclei in relation to the positive control, with a concentration-response relationship and a clear antimutagenic effect. NDI presented statistically significant differences at concentrations of 0.15 and 0.31 $\mu g \cdot mL^{-1}$ when compared to the negative control. There was also a reduction in FMN due to the post-treatment, which was significantly different when compared to the positive control. The difference in NDI was statistically significant only for the concentration of 0.31 $\mu g \cdot mL^{-1}$ when compared to the negative control.

The micronucleus test was used to evaluate antimutagenicity. Pre and post-treatments with extracts and fractions of *E. jambolana* leaves and fruits demonstrated a significant reduction in the frequency of micronuclei for all concentrations when compared to the positive control, suggesting antimutagenic effect of the extracts and fractions tested.

In both treatments (pre and post) and for all extracts and fractions tested, there was a standard concentration-response relationship to the frequency of micronuclei, since higher concentrations presented a mitigation against damage induced by $H_2O_2$, reducing the frequency of micronuclei, and demonstrating that the extracts and fractions from *E. jambolana* may exert action through cellular machinery as possible antimutagenic agents.

Evaluation of the antimutagenicity of fruit extracts and fractions showed that pre-treatment with CEF significantly reduced the frequency of micronuclei when compared to the positive control, whereas NDI did not present a statistically significant difference between the treatments and the negative control results. Post-treatment at different concentrations induced a significant reduction in the frequency of micronuclei in relation to the positive control. A discrete lack of uniformity in nuclear division occurred during NDI, with a statistically significant difference among results for the three tested concentrations when compared to the negative control.

Pre-treatment with HAF significantly reduced the frequency of micronuclei in relation to the positive control, and NDI showed a slight loss of uniformity of nuclear division, with a statistically significant difference at 0.15 $\mu g \cdot mL^{-1}$ when compared to the negative control. A similar profile was observed during post-treatment for the tested concentrations with no loss of uniformity in nuclear division when evaluating NDI results.

DNA protective substances can be classified as either desmutagenics or bioantimutagenics. Desmutagenic agents act directly to inactivate mutagens, or by their precursors irreversibly binding to the mutagenic compound, triggering its chemical inactivation through a direct connection or inhibiting activation by modulation of phase 1 and 2 enzymes. Conversely, bioantimutagenic agents act on physiological mechanisms of DNA protection and repair, inducing mutagenic effect reversal and preventing the fixation of mutations (Kada & Ivbone, 1982). From the results obtained in the comet assay, it is suggested that the mechanism of action of the extracts and fractions, primarily from fruits of *E. jambolana*, is bioantimutagenesis.

However, the fact that pre-treatment with the extracts and fractions evidenced increased genotoxicity induced by $H_2O_2$, indicates that such results might be related to the presence of chemical components in *E. jambolana* test samples which, after metabolism of HepG2 cells, interact with $H_2O_2$ at the time of damage induction.

### 3.4. Chemoprevention capacity by quinone reductase induction

The chemopreventive activity assay used the induction of quinone reductase enzyme activity of *E. jambolana* test samples on Hepa 1c1c7 cell line, which contains amounts of inducible quinone reductase that are easily measurable (Fig. 6).

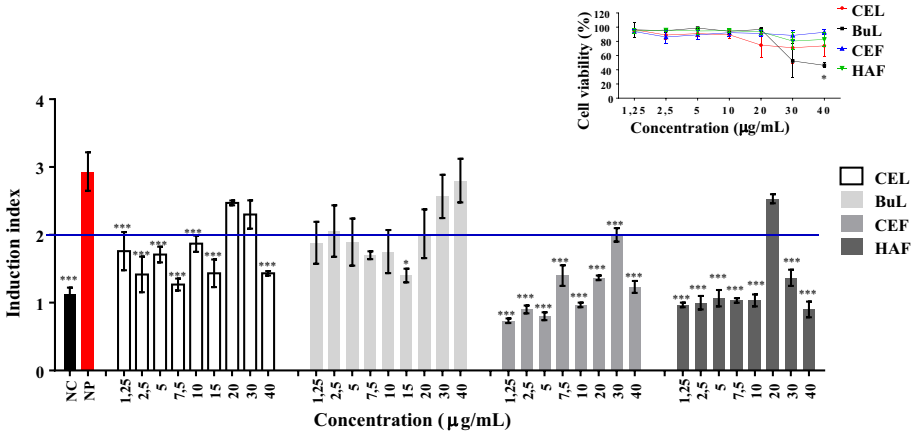



**Fig. 6.** Chemoprevention capacity by Quinone reductase induction assay in Hepa 1c1c7 cell line, after 48 h of treatment with crude extract (CEL), *n*-butanol fraction (BuL) of leaves, crude extract (CEF), hydroalcoholic fraction (HAF) of fruits from *E. jambolana*, culture media as Negative control (NC), and β-naphthoflavone at 0,1 μM as Positive control. Results are demonstrated as mean of Induction index ± standard error (SE). Three independent experiments were analyzed by One-way ANOVA with Dunnett's post-test. The cell viability was measure by crystal violet assay in the same conditions of enzymatic induction assay. The results of samples were compared with positive control (Statistical differences: * indicates $P < 0.05$; ** indicates $P < 0.01$ and *** indicates $P < 0.001$). Statistical analyses were performed with software Graphpad Prism 5.0.

The crude extract from both leaves (CEL) and fruits (CEF) showed induction of quinone reductase activity at concentrations of 20 and 30 μg·mL$^{-1}$, for CEL, and 30 μg·mL$^{-1}$ for CEF. No statistically significant difference was observed in relation to the negative control. The treatment with *n*-butanol fraction of the leaves (BuL) at concentrations of 2.5, 20, 30 and 40 μg·mL$^{-1}$ also showed induction of quinone reductase enzyme. Cell viability levels were observed at 95.1 ± 3.1; 96.8 ± 2.4; 52.4 ± 13.8 and 46.3 ± 3.7% of live cells. Only the highest concentration evidenced a statistically significant difference when compared with the negative control. The hydroalcoholic fraction of fruits (HAF) showed induction of quinone reductase activity only at a concentration of 20 μg·mL$^{-1}$ with 93.3 ± 3.6% of live cells.

The evaluation of quinone reductase activity is a fast and quantitative bioassay extensively used to characterize the chemopreventive activity of various natural and synthetic compounds. Such compounds may be considered active when they are able to induce duplication of enzymatic activity compared to basal activity.

Extracts and fractions of *E. jambolana* promoted the induction of enzymatic activity of quinone reductase to a level more than twice the activity found for the negative control group by using the crystal violet assay. Such extracts and fractions did not demonstrate cytotoxicity for Hepa 1c1c7 cell line.

In conclusion, the purification of extracts from leaves and fruits of *E. jambolana* revealed flavonoids and anthocyanins as major chemical constituents, which might be responsible for their capacity of quinone reductase induction as well as the observed antigenotoxic and antimutagenic effects, directly depending on its concentration. Accordingly, the extracts and fractions from *E. jambolana* may represent prototypes for novel chemopreventive agents. In addition, the inclusion *E. jambolana* fruits in a person's diet may be an alternative for the prevention of diseases such as cancer, as they may be regarded as non-toxic and are already consumed by Brazilian population.

## Conflict of interest

The authors declare no competing financial interest.

## Authors' contributions

Alessandra C. Dametto (PhD) contributed in collecting fruits, running the chemical laboratory work, analyses of the data and drafted the paper. Carenina V. Plaza (Ms) contributed in collecting leaves, running the chemical laboratory work, and analyses of the data. Nivaldo Boralle contributed to NMR analyses, analyses of NMR data, discussion of results, and contributed to critical reading of the manuscript. Dulce H.S. Silva designed the chemical study, supervised the laboratory work and contributed to critical reading of the manuscript. Daniele Agustoni (Ms), Tarsia G. A. Silva (PhD), Thais F. Moreira (PhD) Aline Prieto (PhD) contributed to biological study and analyses of the data. Thais F. Moreira contributed in drafted the paper. Christiane P. Soares designed the biological study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.07.013.

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