Cytogenotoxic and Oxidative Status Evaluation of *Morinda citrifolia*

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Abstract

Cancer is one of the most leading causes of death worldwide. *Morinda citrifolia* was reported to have antitumor effects. Cisplatin (CDDP), Doxorubicin (DOX) and Cyclophosphamid (CPA) are the known effective chemotherapeutics, despite of having several side effects. This study evaluated antitumoral and oxidative effects of the aqueous extract of the fruit of *M. critrifolia* (AEMC) (15, 30, 60 and 120 µg/mL) in comparision to CDDP (1 and 5 µg/mL), CPA (20 µg/mL), DOX (2 µg/mL) and CPA + DOX (20:2 µg/mL) in Sarcoma 180 cells and *Saccharomyces cerevisiae*, respectively. Cytogenetic damage and DNA fragmentation were evaluated with cytokinesis-block micronucleus assay and comet assay, respectively. In addition, *S. cerevisiae* strains were used in oxidative damage evaluation. AEMC induced cytogenetic damage with the increased formation of micronuclei, nuclear buds and nucleoplasmic bridges compared to the antineoplastics tested. AEMC at 120 µg/mL induced significant ($p < 0.05$) DNA damage in Sarcoma 180 cells similar to the CDDP, as well as oxidative damage in *S. cerevisiae* strain deficient in mitochondrial superoxide dismutase (*Sod2Δ*) and cytosolic catalase (*Cat1Δ*). The bioactive compounds present in AEMC such as gallic, caffeic, chlorogenic, ellagic acid and rutin might be responsible for AEMC's antitumoral activity.

Keywords

Cytogenotixicity; *Morinda Citrifolia*; Sarcoma 180; *Saccharomyces Cerevisiae*; Oxidative Damage.

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Introduction

The cancer is one of the most prevalent causes of mortality worldwide [1]. The major problem in the treatment of malignancies involved molecular genetic and epigenetic mechanisms in the development of resistant phenotypes [2], characterizing cancer is a heterogeneous genetic disease with several alterations in different cellular signaling pathways [3].

Chemotherapy is usually well tolerated, but induces necrosis predictive for metastasis risk [4]. The antineoplastics, such as alkylating agents polyfunctional, antimetabolites, antibiotics and mitotic inhibitors induce apoptosis, avoiding inflammatory processes arising from necrosis [5]. Cisplatin (CDDP) induces renal cell death that have been fully ascribed to its ability to generate unrepairable DNA lesions, hence inducing cellular senescence or apoptosis by mitochondrial pathway [6]. Doxorubicin (DOX) is one of the most effective anticancer drugs acting by the inhibition of topoisomerase II, although, its clinical use is limited due to adverse effects, such as cardiotoxicity induced by lipid peroxidation [7] and oxidative stress [8]. Otherwise, Cyclophosphamide (CPA), a nitrogen mustard compound, is a bifunctional alkylating agent, generating DNA adducts, DNA cross-links, and single and double-strand DNA breaks in dividing cells (chromosomal aberrations) [9]. In this context, chemotherapy remains the most important line of defense against hematological malignancies and aggressive forms of solid tumors. Plant compounds have importantly contributed to the discovery of new naturally occurring anticancer agentes [10].

The tropical plant *Morinda citrifolia* Linn. (Rubiaceae) commonly known as "Noni", is widely distributed in Micronesia, Hawaii, Australia, and Southeast Asia [11]. *M. citrifolia* has been used in folk remedies and is reported to have a broad range of therapeutic effects, including antibacterial, antiviral, antifungal, antitumor, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects [12]. Since alternative therapies from plants to fight cancer have presented good examples, this work compared antitumoral and oxidative effects of the aqueous extract from *M. citrifolia* fruits (AEMC) in Sarcoma 180 and *Saccharomyces cerevisiae* cells, respectively.

Methods

Chemicals

The antineoplastics agents Cisplatin (CDDP), Cyclophosphamide (CPA) and Doxorubicin (DOX) were purchase from Eurofarma® Laboratories and dissolved in sterile saline (0.9% NaCl). The combination of both antineoplastic agents Doxorubicin + Cyclophosphamide (AC) were prepared in the same way.

Collection and extraction of plant material

Fruits of *M. citrifolia* were collected in 2014 in the municipality of Altos, Piauí, Brazil, (05° 02' 20" S - latitude, 42° 27' 39" O - longitude) at 187 m above sea level. The botanical identification was held at Center for Environmental Sciences of Tropic Ecotonal Northeast, Teresina, Piauí (volcher number: 21644). After collection, the fruits were dried in a forced air oven for 8 days at a maximum temperature of 45 °C (± 1 °C). Then, they followed by course grinding and were preserved in a amber glass.

HPLC analysis

High performance liquid chromatograph (HPLC) analyses for determination of flavonoids and phenolic compounds were performed on a Waters 2695 liquid chromatograph equipped with autosampler and a variable wavelength UV/VIS detector (Waters 2487 Detector Dual Absorbance, 190-700 nm). Columns: Waters Spherisorb ODS2 (5 µm, 4.6x250 mm). The mobile phases consisted of acetonitrile (A) HPLC grade purchased and 0.1% H_3PO_4 aqueous solution (B, filtered using a Millipore system). The following were the gradient conditions: 5% (A);

15% (A) for 10 min; 35% (A) for 40 min; 100% (A) for 15 min and 5% (A) for 5 min. Total run time was 70 min at a flow rate of 1.8 mL/min. Injection volumes were 20 µL. The AEMC was solubilized in acetonitrile (30%) HPLC grade purchased and 0.1% H_3PO_4 aqueous solution (70%).

Primary culture of Sarcoma 180 cells

Ascite-bearing female mice between 7 and 9 days (post-treated) were sacrificed by cervical dislocation and a suspension of Sarcoma 180 (S180) cells was harvested from the intraperitoneal cavity under aseptic conditions. The suspension was centrifuged at 500 X g for 5 min to obtain a cell pellet and washed three times with RPMI medium. Cell concentration was adjusted to 0.5 x 10^6 cells/mL in supplemented RPMI 1640 medium with 20% foetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Grand Island, NY), and incubated with CDDP (1 and 5 µg/mL), DOX (2 µg/mL), CPA (20 µg/mL), DOX + CPA at same concentration (2:20 µg/mL) and AEMC (15, 30, 60 and 120 µg/mL) for 72 h at 37 °C with 5% CO_2. The protocols were approved by the Ethical Committee on Animal Research (Process No. 081/2014) and followed the Brazilian (*Colégio Brasileiro de Experimentação Animal* - COBEA) and International Standards on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council).

Trypan blue exclusion test

Cell viability was analyzed by the application of Trypan blue exclusion test according to Renzi et al. [13]. After 72 h, 10 µL of Trypan Blue were added to 90 µL of S180 cell suspension from each culture and analyzes were performed under an optical microscope using Neubauer chamber.

Cytokinesis-Block Micronucleus Assay (CBMN)

For this study, criteria for the identification of binucleate cells and CBMN assay parameters are described by Fenech et al. [14]. S180 cells suspension (0.5 x 10^6/mL) was added in supplemented RPMI 1640 medium as described above and phytohemagglutinin A (Gibco, Grand Island, NY). Then, each 24-well plate was treated as previously mentioned. Cells were incubated for 44 h at 37°C with 5% CO_2. After incubation, 6 µg/mL of cytochalasin B (Sigma, St. Louis, MO) was added to the cultures. Plates were maintained at incubator for additional 28 h. At the end of these two incubation phases, the cultures were centrifuged at 800 rpm for 5 minutes. Then, the supernatant was removed and cell pellets were gently agitated with 5 mL of fixing solution (methanol: acetic acid 5:1). After centrifugation and repeating fixing processes, the supernatant was discarded and 2 to 4 drops of cell suspension was dripped on slides, which were stained with Giemsa 5%. The previously coded slides were examined in a blind test with an optical microscope at magnification of 100X and a total of 1000 cells/slide in duplicate was analysed for cytogenetic damage.

Analyzes of DNA damage by comet assay

The comet assay was performed according to Speit and Rothfuss [15]. The slides were previously coated with a layer of agarose with normal melting point (NMPA; 0.75%) in phosphate buffered saline (PBS; pH 7.4) and stored in a darkroom, wet at 4°C until test commenced (24 h). After incubation in same concentrations described above, 10 µL of S180 cell suspension were added to 90 µL of low melting point agarose (LMPA; 1.5%) at 35°C, the mixture was added to NMPA pre-coated slides and covered with cover slips. After solidification, slides were immersed in lysis solution (10% DMSO, 1% Triton X-100 mM Tris, 2.5 M NaCl, 100 mM EDTA, pH 10.0 to 10.5) for 72 h at 4°C. After the elapsed time, slides were then rinsed with DW, placed horizontally in a electrophoretic tank and covered with fresh electrophoresis buffer (0.0075 M EDTA 1 mM, pH 13) for 20 min. Electrophoresis

was performed for 15 min at 300 mA and 25 V then followed by neutralization (400 mM Tris; pH 7.5), fixation (15% v/v trichloroacetic acid, 5% v/v of zinc sulfate, 5% glycerol), washing with DW following to over-night drying at 18°C. The gels were then rehydrated for 5 min in DW and stained for 15 min (37°C) with staining solution {1 part of solution B (0.2% v/v ammonium nitrate, 0.2% silver nitrate v/ v, 0.5% v/v tungstosalisilic acid, 0.15% v/v formaldehyde, 5% v/v sodium carbonate) and 3 part solution A (sodium carbonate to 5%)}. Stop solution was prepared with a solution of 1% acetic acid gels and subjected to dry at room temperature. Finally, a total of 100 cells were analyzed randomly per treatment. Two parameters were considered, such as index of damage (ID; 0-400) and frequency of damage (FD; 0-100%). For the calculation of identity, the cells were visually classified into five categories according to the size of comet tails (0 = no tail, 4 = maximum length of the tail), which resulted in a DNA damage score for each sample and hence for each group. Frequency of damage (FD) in percentage was calculated for each sample based on the number of cells with tails compared to damaged cells with zero (0). All slides were coded for blind analysis.

Oxidative damage in *Saccharomyces cerevisiae* cells

For oxidative studies, wild-type strain EG103 (SOD) *S. cerevisiae* and isogenic strains: deficient strains in cytosolic (*Sod1Δ*) and mitochondrial superoxide dismutase (*Sod2Δ*), double mutant (*Sod1ΔSod2Δ*), catalase (*Cat1Δ*), and cytosolic superoxide dismutase + catalase (*Cat1ΔSod1Δ*) were used and kindly provided by E.B. Gralla (University of California, Los Angeles, Los Angeles, CA). The strains were cultured in YEL medium (yeast extract 0.5%, 2% of Bacto-peptone and 2% of glucose) at 28°C in an orbital stirrer until it reached the stationary growth phase [16]. Cells in suspension were seeded from the center to the edge of a Petri dish in a continuous cycle on both sides, containing in the center of plate a sterile filter paper disk which received CDDP (1 and 5 μg/mL), CPA (20 μg/mL), DOX (2 μg/mL), DOX + CPA (AC) (2:20 μg/mL) and AEMC (120 μg/mL). After 48 h of incubation at 30°C, the zones of inhibition growth were measured in millimeters (mm). The assays were performed in duplicate.
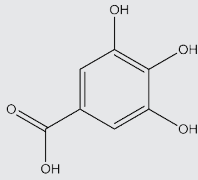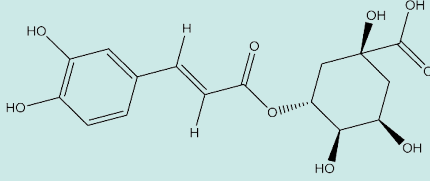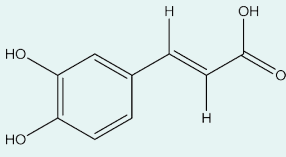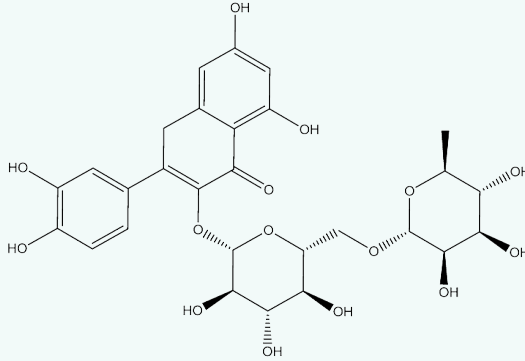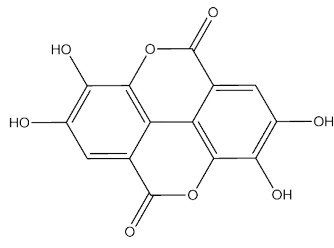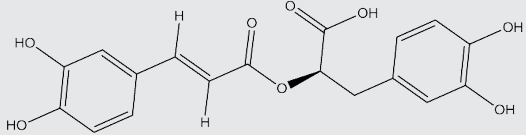
Statistical analysis

In order to determine differences among treatments, data expressed as mean ± standard error of the mean (S.E.M.) were compared by one-way analysis of variance (ANOVA) followed by the Tukey test ($p < 0.05$) using the Graphpad program (Intuitive Software for Science, San Diego, CA). All studies were carried out in duplicate represented by independent biological evaluations.

Results

HPLC analysis

The correlation of chromatographic peaks was achieved by comparing of experimental retention times (t_R) with reference standards **(Table 1)**. All chromatographic preliminary phytochemical analysis of AEMC were carried out in triplicate at room temperature and revealed phenolic compounds (gallic acid, chlorogenic acid, caffeic acid, ellagic acid and rosmarinic acid) and flavonoid (rutin) with the following t_R: 3,4,5-trihydroxybenzoic acid(1) t_R= 5 min; (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1 carboxylic acid (2) t_R = 14,9 min; (E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid (3)t_R= 15,1 min; 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5 trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one (4)t_R = 32.5 min; 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione (5)t_R = 35 min; (2R)-

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Table 1. HPLC analysis of aqueous extract of the fruit of *Morinda citrifolia*.

Compounds	Chemical structures	Retention time (t_R)
Gallic acid		87.50
Chlorogenic acid		62.50
Caffeic acid		68.75
Rutin		62.50
Ellagic acid		81.25
Rosmarinic acid		87.50

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3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydro-xyphenyl)prop-2-enoyl] oxypropanoic acid (6)t_R = 37.5 min.

Cell viability

S180 cells viability was evaluated after 72 h following ex vivo exposure to antineoplastics and AEMC. S180 cells treated with CDDP (1 and 5 µg/mL), CPA, DOX, AC and AEMC (30, 60 and 120 µg/mL) showed cell viability reduction (48.7 ± 2.5, 39.2 ± 1.5, 39.5 ± 2.1, 45.5 ± 2.5, 38.5 ± 3.6, 54 ± 2.3, 53.7 ± 2.5 and 50.7 ± 1.3, respectively) when compared with untreated cells (64.5 ± 3.5) (**Figure 1**, p<0.05).

Apoptosis and necrosis induced by antineoplastics and AEMC in S180 cells

CDDP (1 and 5 µg/mL), CPA, DOX, AC and AEMC (30, 60 and 120 µg/mL) induced apoptosis in S180 cells (444 ± 40.2, 612 ± 103.2, 312 ± 25.4, 283 ± 6.3, 553 ± 18.3, 324.5 ± 66.5, 367.5 ± 67.1 and 562 ± 55.1, respectively) in comparison with negative control (42.5 ± 6.3) and AEMC at 60 and 120 µg/mL produced similar results to the anticancer agents (**Figure 2**, p<0.05). Meanwhile, all antineoplastics substances also induced the arising of necrosis figures [75 ± 7.1, 81.5 ± 3.5, 107 ± 10.6, 76.5 ± 4.9, 91 ± 8.5, 57.5 ± 10.6, 58 ± 16.9 for CDDP (1 and 5 µg/mL, CPA, DOX, AC and AEMC (60 and 120 µg/mL) respectively (p<0.05)], though these findings were not in lower concentrations of AEMC (p>0.05).

Nuclear abnormalities induced by antineoplasics and AEMC in S180 cells

Micronucleus were statistically more found at 5 µg/mL of CDDP (39.4 ± 1.5), at 120 µg/mL of AEMC (21.5 ± 2.1) and in sarcoma cells treated with DOX (14.2 ± 1.5) and with the association of AC (19.2 ± 2.8) when compared to the negative group (2.2 ± 1.4) (**Figure 3**). On the other hand, only CDDP (5 µg/mL) and AEMC at concentration of 120 µg/mL induced significant increasing of nucleoplasmic bridges (25.3 ± 7.1 and 18.5 ± 2.1) (p<0.05).

Once again, CDDP (1 and 5 µg/mL), CPA and DOX + CPA (AC) also increased the number of chromosomal abnormalities (21.5 ± 4.9, 49.5 ± 3.5, 19.5 ± 2.1 and 21.4 ± 1.5). However, only the highest concentration of AEMC was able to induced the formation of nuclear buds (39.5 ± 0.7) (**Figure 4**, p<0.05).

Figure 1: Effect of aqueous extract of fruit of *M. citrifolia* (AEMC) on Sarcoma 180 cell viability determined by trypan blue staining after 72 h of incubation. (A) Number of viable cells; (B) number of non-viable cells; negative control (NC). Cisplatin (CDDP), Cyclophosphamide (CPA) 20 µg/mL, Doxorubicin (DOX) 2 µg/mL and Doxorubicin + Cyclophosphamide (AC) 20:2 µg/mL was used as positive control. Results are expressed as mean ± standard error of mean (SEM) from three independent experiments. *p<0.05 compared to control by ANOVA followed by Tukey test.

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SECTION: TOXICOLOGY & THERAPEUTICS
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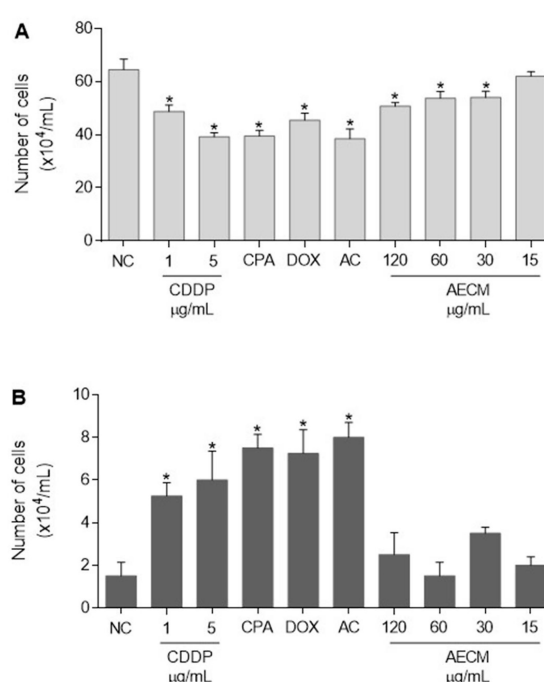
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Vol. 9 No. 96
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Figure 2: Cytotoxicity in Sarcoma 180 cells treated with aqueous extract of fruit of *M. citrifolia* (AEMC). NC: negative control. CDDP: cisplatin. CPA: cyclophosphamide (25 µg/mL). DOX: doxorubicin (2 µg/mL). AC: Doxorubicin + Cyclophosphamide (2:20 µg/mL). *p<0.05 compared to the negative control by ANOVA followed by Tukey test.

Photomicrograph profile of apoptotic (A) and necrotic (B) cell marked by asterisk. Giemsa 10% (100X).

Figure 3: Mutagenicity in Sarcoma 180 cells treated with aqueous extract of fruit of *M. citrifolia* (AEMC). NC: negative control. CDDP: cisplatin. CPA: cyclophosphamide (25 µg/mL). DOX: doxorubicin (2 µg/mL). AC: Doxorubicin + Cyclophosphamide (2:20 µg/mL). MNBN: micronucleated binucleated cell. *p<0.05 compared to the negative control by ANOVA followed by Tukey test.

Photomicrograph profile of micronuclei (A) and binucleated (B) cell with nucleoplasmic bridge marked by asterisk. Giemsa 10% (100X).

Figure 4: Chromosomal abnormalities in Sarcoma 180 cells treated with aqueous extract of fruit of *Morinda citrifolia* (AEMC). NC: negative control. CDDP: cisplatin. CPA: cyclophosphamide (25 µg/mL). DOX: doxorubicin (2 µg/mL). AC: doxorubicin + cyclophosphamide (2:20 µg/mL). *p<0.05 compared to the negative control by ANOVA followed by Tukey test.
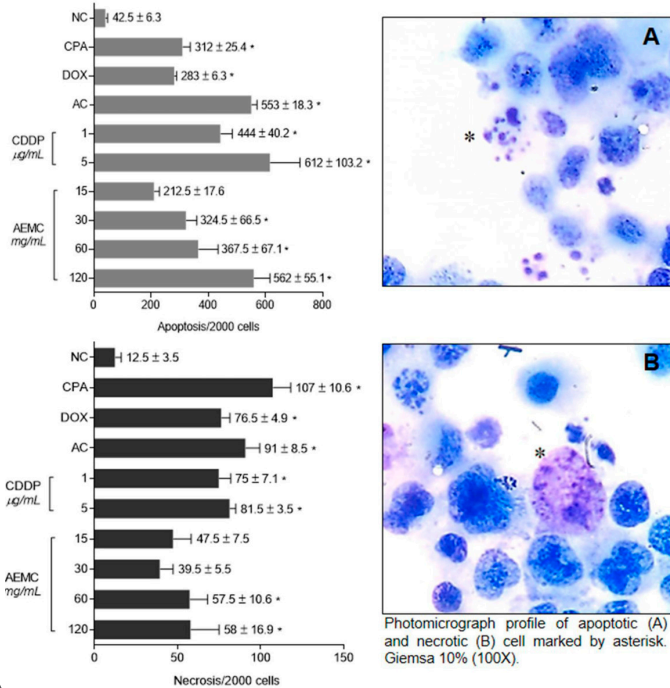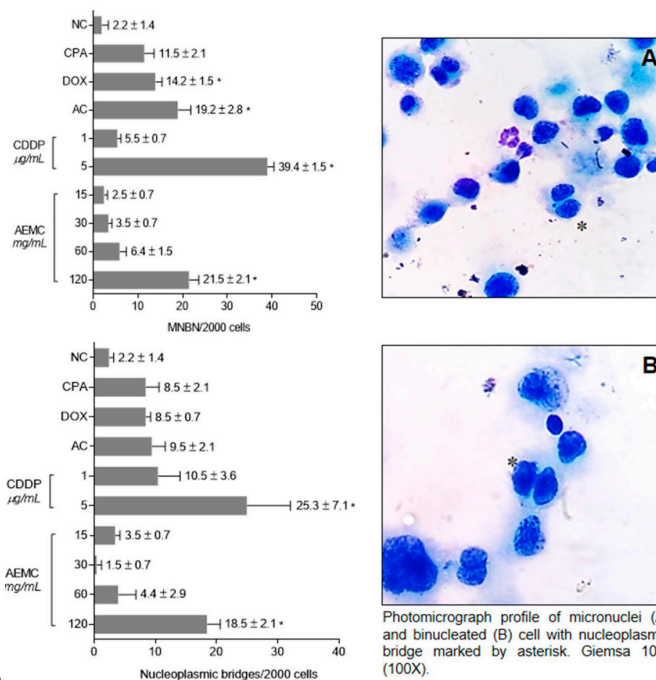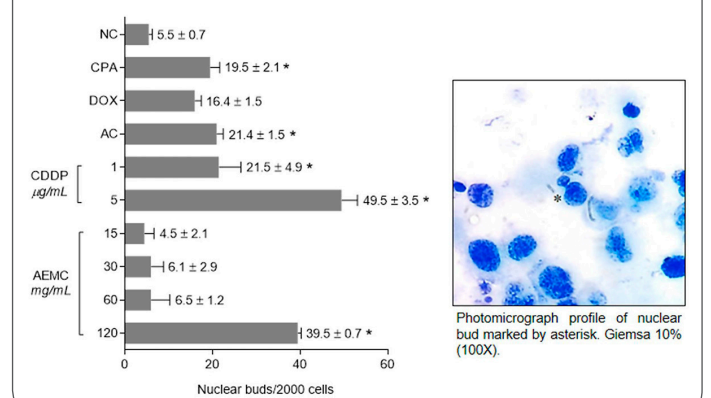

Photomicrograph profile of nuclear bud marked by asterisk. Giemsa 10% (100X).

INTERNATIONAL ARCHIVES OF MEDICINE
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Vol. 9 No. 96
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Tabl 2. Inhibition growth induced by antineoplastics and aqueous extract of fruit of *M. citrifolia* (AEMC) in *Saccharomyces cerevisiae* strains.

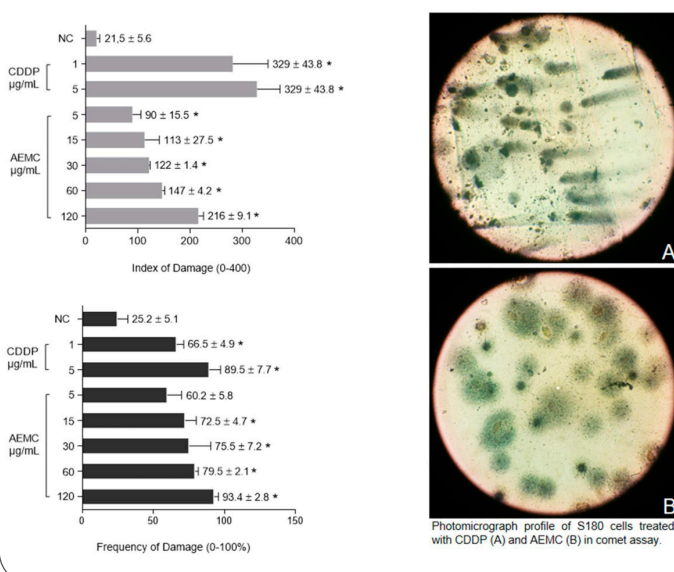| Treatments	Strains					
	SODWT	**Sod1Δ**	**Sod2Δ**	**Sod1ΔSod2Δ**	**Cat1Δ**	**Sod1ΔCat1Δ**
Saline 0.9%	2.2 ± 0.9	2.7 ± 1.0	3.0 ± 0.8	3.0 ± 0.8	2.5 ± 0.5	2.2 ± 1.0
H_2O_2 10 mM	21.2 ± 3.5*	22.1 ± 2.2*	20.5 ± 3.1*	20.8 ± 3.5*	22.5 ± 2.1*	21.4 ± 3.2*
CPA 20 μg/mL	23.5 ± 1.3*	27.5 ± 1.7*	34.2 ± 3.0*	35.7 ± 1.0*	36.2 ± 1.2*	34.2 ± 1.0*
DOX 2 μg/mL	10.7 ± 2.2*	10.2 ± 1.2*	11.3 ± 1.4*	9.5 ± 2.0*	12.2 ± 1.5*	10.4 ± 1.6*
AC 20:2 μg/mL	10.5 ± 2.0*	10.2 ± 0.8*	11.2 ± 1.7*	10.4 ± 1.4*	10.2 ± 2.0*	11.2 ± 1.0*
CDDP 1 μg/mL	6.0 ± 1.4*	4.7 ± 1.2*	10.2 ± 2.4*	6.5 ± 2.5*	8.5 ± 2.7*	6.7 ± 2.5*
CDDP 5 μg/mL	9.2 ± 1.5*	9.2 ± 2.5*	9.5 ± 2.5*	10.5 ± 2.6*	9.5 ± 2.5*	11.4 ± 2.5*
AEMC 120 μg/mL	6.5 ± 1.7	5.7 ± 1.0	8.5 ± 1.2*	6.4 ± 1.4	10.2 ± 2.0*	7.2 ± 1.0

#: values represent mean ± standard error of inhibition growth of *S. cerevisiae* strains. H_2O_2: hydrogen peroxide. CPA: Cyclophosphamide. DOX: Doxorubicine. AC: Doxorubicin + Cyclophosphamide. CDDP: cisplatin. ANOVA followed by Tukey test. *p<0.05 compared to Saline.

DNA damage induced by antineoplastics and AEMC in S180 cells

All concentrations of the AEMC increased the ID after 72 h of incubation (90 ± 15.5, 113 ± 27.5, 122 ± 1.4, 147 ± 4.2 and 216 ± 9.1 for 5, 15, 30, 60 and 120 μg/mL, respectively) (**Figure 5**, p<0,05) when compared to negative control (21.5 ± 5.6). Meanwhile, the positive control CDDP presented ID values of 282 ± 47.5 and 329 ± 43.8 for 1 and 5 μg/mL, respectively. Regarding to FD, CPPD also caused significant increasing (66.5 ± 4.9 and 89.5 ± 7.7, respectively) and only the lowest concentration of the AEMC did not induce FD increasing (60.2 ± 5.8, p>0.05) (**Figure 5**).

Figure 5: Genotoxicity in Sarcoma 180 cells treated with aqueous extract of fruit of *M. citrifolia* (AEMC). NC: negative control. CDDP: cisplatin. *p<0.05 compared to the negative control by ANOVA followed by Tukey test.

Photomicrograph profile of S180 cells treated with CDDP (A) and AEMC (B) in comet assay.

Evaluation of oxidative damage induced by antineoplastics and AEMC in *S. cerevisiae* strains

All antineoplastics tested (CDDP, CPA, DOX and AC) induced oxidative damage in *S. cerevisiae strains* (SODWT, *Sod1Δ*, *Sod2Δ*, *Sod1ΔSod2Δ*, *Cat1Δ* and *Cat1ΔSod1Δ*). On the other, AEMC at 120 μg/mL induced oxidative damage only in the strain with mutation for mitochondrial superoxide dismutase (*Sod2Δ*) and catalase (*Cat1Δ*) (**Table 2**).

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SECTION: TOXICOLOGY & THERAPEUTICS
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Vol. 9 No. 96
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Discussion

Cancer is represented by a pool of diseases related to the accumulation of multiple genetic changes that leads to genetic instability (DNA damage) and repair failures and they have been associated with the diagnosis and prognosis of neoplasms. Such instability can be mediated through chromosomal changes, that can be detected by, cytogenetical tecniques [17]. Assays as CBMN, Single Cell Gel Electrophoresis and oxidative analyzes in *S. cerevisiae* strains have been proposed as biomarkers to assess cytogenetic alterations and measure DNA damage [18].

Primary cultures of tissues that preserve the original tumor microenvironment injected into mice has been used as models of human sarcomas [19], allowing the understanding of the molecular biology of cancer and cellular changes after exposure to antiproliferative agents. Some tools, such as Cytokinesis-Block Micronucleus Assay (CBMN), a multi-endpoint assay that measures chromosomal damage; micronuclei (MN), reflecting chromosome breaks; nucleoplasmic bridges (NPB), reflecting chromosomal rearrangements and nuclear buds (NBUD), reflecting gene amplification, along with other cellular events such as apoptosis and necrosis [14] are often used to evaluate pharmacological and toxicological effects of bioactive natural and synthetic compounds in cellular models.

S180 cells provide a paradigm to identify unknown pharmacogenetic variants associated with drug-induced cytotoxicity [20]. Herein, all chemotherapics used (CDDP, CPA, DOX, AC) and AEMC induced apoptosis, necrosis and reduced cell viability of sarcoma 180 cells.

CDDP is capable to induce adduct formation effects and peculiar DNA-depentent functions, including inhibition of replication and transcription, cell cycle arrest, and DNA damage that can lead to the cellular death and apoptosis, thus may followed by mutations [21]. Our results suggest that genetic factors involved in cytotoxicity also contribute to cisplatin-induced apoptosis. CDDP have been fully ascribed to its ability to generate unrepairable DNA lesions, hence inducing either a permanent proliferative arrest known as cellular senescence or apoptosis induction by mitochondrial pathway [22].

CPA is metabolically activated by the enzyme CYP2B6 and induced cell death via formation of DNA adducts by it's active metabolite, phosphoramide mustard. These adducts are the most physiologically relevant because of their ability to block the replication of DNA and its association with cytotoxicity [23]. These DNA damages presented as nuclear abnormalities, such as micronuclei and formation of nuclear buds, were also found after treatment with CDDP (5 µg/mL), AEMC (120 µg/mL), DOX and with the association of AC. However, only CDDP (5 µg/mL) and AEMC (120 µg/mL) induced significant increasing of nucleoplasmic bridges. Moreover, all concentrations of the AEMC increased the ID and FD after 72h of incubation, but only the lowest concentration did not induce FD increasing.

Cytotoxicity is a broad phenotype in which antitumor effects may include the induction of apoptosis as seen with AEMC-Sarcoma treated cells. Some compounds presented in the AEMC, as the chlorogenic acid (CGA), a family of polyphenolic esters, has been attributed in the amplification antitumor effects of 5-FU in human hepatocarcinoma cells by inhibition of extracellular signal-regulating kinases. Other compounds also found in the AEMC, such as gallic, caffeic and ellagic acids have also been reported as cytotoxic apoptotic mechanisms. Gallic acid shows cytotoxic effects due to the presence of phenolic hydroxyl groups and carboxyl groups which may involve the specific cytotoxicity between normal and tumor cells. Moreover, it decreases cell viability in a dose dependent manner in DU145 and 22Rv1 cell lines by induction of apoptosis-related mechanisms [24]. Meanwhile, caffeic acid has been related to induction of cell

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2016

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cycle arrest and apoptosis, protein kinases changes and inhibition of COX-2 activity [25] and ellagin acid induces apoptosis in human pancreatic adenocarcinoma and hepatocarcinoma (HepG2) cells [26], sugesting mechanism involving mitochondrial membrane depolarization, release of protein C and caspases activation [27], activation of transcription factors and anti-apoptotic proteins dysregulation [28].

In a similar way, the flavonoid rutin also plays antitumor activity by induction of DNA damage in cells mutant for BRCA gene, cell cycle arrest (G2/M) in neuroblastoma cell lines by apoptosis-inducing mechanism, as well as regulation of gene expression suppressing tumor [29]. These are major bioactive constituents of AEMC responsible for various pharmacological activities [30].

Necrosis was also identified in antineoplastics- and AEMC-sarcoma treated cells. In toxic stimulus analyzes, higher doses regularly develops concomitant features of apoptosis and necrosis. Under such conditions, severity and not specificity of the stimulus selectively determines how cell death occurs. If the necrosis prevails, early lesions on the plasma membrane occur instead of cell shrinking [21]. Therefore, depending on the concentration used, many different processes may be influenced and/or altered, suggesting that dose-dependent regulation of cellular process reflects signalization triggered by bioactive compounds as stated here and by others. For example, previous studies with the diterpenoid casearin X also reported characteristics of apoptosis or secondary necrosis [31].

Micronuclei, biomarkers of genotoxic events and chromosomal instability were induced by AEMC (120 μg/mL), while the other nuclear abnormality, such as NPB, were produced by AECM and CDDP. The MN is frequently originated during anaphase from lagging acentric chromosome or chromatid fragments caused by misrepair of DNA breaks or unrepaired DNA breaks. Mal-segregation of whole chromosomes at anaphase may also lead to MN

formation as a result of hypomethylation of repeat sequences in centromeric and pericentromeric DNA, defects in kinetochore proteins or assembly, dysfunctional spindle and defective anaphase checkpoint genes. NPB originates from the dicentric chromosomes, may occur due to misrepair of DNA breaks, telomere end fusions, and could also be observed when defective separation of sister chromatids at anaphase due to failure of decatenation. NBUD represents the process of elimination of amplified DNA, DNA repair complexes and possibly excess chromosomes from aneuploid cells [14].

The yeast *S. cerevisiae* also provides a powerful experimental instrument for the study of drugs' effect on eukaryotic cells [32]. Herein, oxidative damage in *S. cerevisiae* point out that the AEMC (120 μg/mL) induced oxidative damage similar to CDDP, DOX and AC at the concentrations tested to the strains *Sod2Δ* and *Cat1Δ*. Hung et al. [33] have suggested that caffeic acid, presented in AECM, reduces mitochondrial membrane potential and induces apoptosis [34].

CDDP-induced oxidative stress is thought to be mediated by several mechanisms which include mitochondrial dysfunction and GSH depletion [35]. Oxidative damage has been observed *in vivo* following exposure to CDDP in several tissues, suggesting a role for oxidative stress in the pathogenesis of CDDP-induced dose-limiting toxicities. CDDP induced a mitochondrial-dependent ROS response that significantly enhanced the cytotoxic effect caused by DNA damage. However, ROS generation is independent of the amount of CDDP-induced DNA damage [36]. CDDP has been used in studies on drug mechanisms in yeast. Studies in *S. cerevisiae* and *S. pombe* have focused on the toxic effects of CDDP in DNA repair mutants and the mechanisms of repair of CDDP-induced DNA lesions [37]. Similarly, CPA is widely used in the treatment of tumors through production of ROS, which play an important role in cytotoxicity-mediated apoptosis [38]. DOX indu-

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ces cardiotoxicity by lipid peroxidation [7] caused by oxidative stress [8].

Despite of these genotoxic effects, *M. citrifolia* has been used in traditional therapies against several chronic diseases, including cancer. Damnacanthal, a compound isolated from *M. citrifolia*, increases anti-tumorigenic activity in human colorectal cancer cells [39]. Futhermore, pre-clinical studies have suggest that Noni may have antitumor proper¬ties due to the immune system stimulating using macrophages, natural killer and T cells dependent ways [40].

It is likely that chemicals presented in AECM, such as gallic acid, chlorogenic acid, caffeic acid, ellagic acid and rosmarinic acid and rutin, are involved in the cytotoxicity on sarcoma and *S. cerevisiae* mutants cells, by direct ou indirect (oxidative) DNA damages, leading to apoptosis. *In vitro* and *in vivo* investigations are in progress with sarcoma 180 models to detail the mechanisms(s) of action.

Conclusion

The aqueous fruit extract of *Morinda critrifolia* (AEMC) induced cytogenetic damages with increasing in the formation of micronuclei, nuclear buds and nucleoplasmic bridges like the antineoplastics, Cisplatin, Cyclophosphamide and Doxorubicin taken as standards in Sarcoma 180 cells. Additionally, AEMC also exhibited a Cisplatin like oxidative damage in mitochondrial superoxide dismutase and cytosolic catalase dificient *Saccharomyces cerevisiae* strains. Phytochemicals, present in AEMC fruit such as gallic, caffeic, chlorogenic and ellagic acid and flavonoid rutin may be responsible for the antitumoral effects. Furtther researches are recommended to find out the responsible chemical moity(s) with the appropriate action mechanism(s) of our found activities.

Conflict of interest

None declared.

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