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In Vitro Evaluation of the Genotoxic Activity and Apoptosis Induction of the Extracts of Roots and Leaves from the Medicinal Plant *Coccoloba mollis* (Polygonaceae)

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ABSTRACT *Coccoloba mollis* (Family Polygonaceae) is a medicinal plant popularly used in cases of memory loss, stress, insomnia, anemia, impaired vision, and sexual impotence, but the scientific literature, to date, lacks studies on the biological effects of this species, particularly with regard to cytotoxicity and induction of DNA damage. The aim of the present study was to assess *in vitro* (in hepatic HTC cells) ethanolic extracts of the roots and leaves of *C. mollis* for cytotoxicity, genotoxicity, and induction of apoptosis. For these evaluations the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay, comet assay, micronucleus test with cytokinesis block, and an *in situ* test for detection of apoptotic cells with acridine orange staining were used. The results showed that the extract obtained from the roots of *C. mollis* is more cytotoxic than that obtained from the leaves and that the reduction in cell viability observed in the MTT assay was a result, at least in part, from the induction of apoptosis. Both extracts induced DNA damage at a concentration of 20 μg/mL in the comet assay, but no genotoxicity was detected with any of the treatments carried out in the micronucleus test.

KEY WORDS: • ***apoptosis*** • **Coccoloba mollis** • ***cytotoxicity*** • ***genotoxicity*** • ***herbal extract*** • ***Polygonaceae***

INTRODUCTION

Medicinal plants constitute a source of raw material for traditional and modern medicine.[1] Currently, they are used worldwide as home medicines, can be sold directly to the public without prescription, and even provide compounds for the pharmaceutical industry. In both 2001 and 2002, approximately one-quarter of the bestselling drugs worldwide were natural products or derived from natural products.[2] In Brazil, medicinal plants are widely used in rural and urban areas,[3] but little is known or recorded about the popular healing culture involving medicinal plants.[4]

The interest of the public and scientific community in the benefits of natural products or their active components has increased in the last few decades.[5] Large-scale screenings of medicinal plants have identified numerous favorable phytochemicals to human health[6]; otherwise, adverse effects can result from the absence of regulation and unknown interactions with other drugs.[7] Special attention must be paid to evaluation of the safety, efficacy, and quality of natural products. For this evaluation national and international regulatory agencies recommend the determination of genotoxicity/mutagenicity of natural therapeutic agents.[8,9]

Coccoloba mollis Casaretto (Family Polygonaceae) is a species that has being prescribed as a phytotherapeutic in Brazil under the name "*Erva da memoria*" or memory herb. This food is prepared in a craft manner with the roots and leaves of the plant, extracted with alcohol and protected from light for about 15 days. It is recommended that users ingest 10 drops of this tincture orally, twice or three times per day, diluted in water (tea). *C. mollis*, commonly known as "*folha-de-bolo*" or "*falso-novateiro*," is a 4–12-m tall tree and occurs in shrublands and semideciduous forests of the Parana River basin, in the southcentral region of Brazil.[10] It has been popularly used in cases of memory loss, stress, insomnia, anemia, vision impairment, and sexual impotence. The species of this genus have been used by the Brazilian population as an astringent, in the treatment of fever, diarrhea, gonorrhea, hemorrhoids, menstrual problems, and uterine hemorrhage.[11,12] However, they have been little studied, and there are no reports in the scientific literature to date on the biological effects of these species in *in vitro* and *in vivo* systems. Thus, the aim of the present work was to determine the cytotoxicity, genotoxicity, and ability to induce apoptosis of the ethanol extracts of leaves and roots of *C. mollis* in an *in vitro* test system (HTC cells), in order to contribute to the scientific investigation of medicinal plants traditionally used by the public.

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MATERIALS AND METHODS

Extracts of C. mollis

Dried and powdered material (roots and leaves) of *C. mollis* was extracted with 95% ethanol at room temperature, and the solvent was removed under vacuum to yield 40 g of root extract (RE) and 85 g of leaf extract (LE). RE was subjected to successive fractional partition by *n*-hexane and ethyl acetate. The hexane-soluble fraction (4.5 g) was chromatographed on a silica gel (163.66 g) column using solvents of increasing polarity (*n*-hexane, dichloromethane, ethyl acetate).The leaf material was subjected to successive fractional partition by ethyl acetate; these fractions (30 g) were chromatographed on a silica gel (174 g) column using solvents of increasing polarity (*n*-hexane, dichloromethane, ethyl acetate). The major compounds of the extracts identified through spectroscopy methods ($^{1}H/^{13}C$ nuclear magnetic resonance, gas chromatography-mass spectrometry, infrared) were two anthraquinones (emodin and physcion) from the root ethanolic extract and a mixture of long-chain hydrocarbons, carboxyl esters, and 3-taraxerone from the leaf ethanolic extract. Phytochemical screening using pharmacognostic methodology revealed the presence of flavonoids and tannins in roots and leaves, whereas this analysis yielded negative results for alkaloids, coumarins, saponins, and simple phenolics.[13,14] The vegetal material collected was identified by Ana Odete Santos Vieira, Ph.D. from the Department of Animal and Vegetal Biology of the Universidade Estadual de Londrina, Londrina, PR, Brazil, and a voucher specimen was deposited under the identification number Barros, I B 001.

For use in culture, each of the extracts was dissolved in dimethyl sulfoxide and phosphate-buffered saline or Dulbecco's modified Eagle's medium/F12 culture medium (Gibco, Grand Island, NY, USA) without serum [in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay], where the dimethyl sulfoxide concentration did not surpass 10% in the stock solution. Afterward, the solutions were filter-sterilized using a disposable filtration unit of 0.22 μm porosity (Millex®, Millipore, Bedford, MA, USA).

DNA damage and apoptosis-inducing agents

To induce DNA damage in the comet and micronucleus assays, benzo[*a*]pyrene (CAS 50-32-8; Fluka, Buchs, Switzerland) was used at a final concentration of 25 μg/mL in culture medium, for 24 hours and 26 hours, respectively. Induction of apoptosis was tested with cisplatin (CAS 15663-27-1; Sigma Aldrich, St. Louis, MO, USA) at a final concentration of 6 μg/mL for 24 hours.

Cell line

HTC cells (proficient in drug metabolism) were obtained from the Banco de Células do Rio de Janeiro, Universidade Federal de Rio de Janeiro, Rio de Janiero, RJ, Brazil. They were cultivated in a BOD (biochemical oxygen demand) incubator (FANEM®, São Paulo, SP, Brazil) at 37°C in 25-cm^2 culture flasks containing 5 mL of Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum (Gibco) and antibiotic/antimycotic. The genotoxicity tests were conducted with approximately 10^6 cells seeded per experimental culture flask. In the apoptosis assay, the cells (approximately 2.5×10^5) were grown on 20-$\times$20-mm coverslips in 10-cm^2 culture tubes containing 3 mL of culture medium.

MTT cytotoxicity assay

The cytotoxicity assay was performed according to the protocol described by Mosmann[15] with some modifications. Approximately 2.5×10^4 HTC cells were seeded in each well of a microplate and then treated with the extracts for 24, 48, and 72 hours. At the end of this period, cells were incubated with MTT (CAS 298-93-1; Sigma Aldrich; 0.005 g/5 mL of phosphate-buffered saline; 10 mL of culture medium without serum) for 4 hours. Absorbance was measured with a spectrophotometer plate reader (Uniscience, São Paulo) at 550 nm.

The RE and LE concentrations tested in the MTT assay were 0.2 μg/mL (RE1 and LE1), 2 μg/mL (RE2 and LE2), 20 μg/mL (RE3 and LE3), 200 μg/mL (RE4 and LE4), and 2,000 μg/mL (RE5 and LE5) in culture medium. Genotoxicity (comet assay and micronucleus test) were determined using three concentrations defined by the MTT results.

Comet assay (single cell gel electrophoresis [SCGE])

The comet test (SCGE) was carried out based on the conditions of Tice *et al.*[16] with three concentrations of the extracts defined by MTT as noncytotoxic (0.2 μg/mL, 2 μg/mL, and 20 μg/mL). In brief, after 24 hours of treatment (DNA damage-inducing agent, RE, or LE), HTC cells were trypsinized (500 μL of 0.025% trypsin-EDTA, 37°C), the cell suspension was centrifuged (5 minutes, 215 *g*), and the pellet was resuspended in 500 μL of culture medium. Next, 120 μL of low-melting-point agarose (0.5%) was added to the cells, where this mixture was deposited on pregelatinized slides (normal-melting-point agarose, 1.5%) and placed in lysis solution for at least 1 hour. After denaturation (20 minutes) and alkaline electrophoresis (25 V, 300 mA, 20 minutes), the slides were neutralized, fixed, and kept refrigerated until the time of analysis. The slides were stained with ethidium bromide and analyzed visually[17] with a fluorescence microscope (excitation filter of 420–490 nm and emission filter of 520 nm) at$\times$400 magnification. The comets were classified as follows: Class 0, nucleus without tail; Class 1, nucleus with tail less than the diameter of the nucleus; Class 2, nucleus with tail one to two times the diameter of the nucleus; and Class 3, nucleus with tail more than two times the diameter of the nucleus. The experiments were performed in triplicate, and 300 cells were analyzed per treatment. Determination of cell viability was carried out by the trypan blue exclusion method, where only treatments with greater than 80% viability were considered.

Micronucleus test with cytokinesis block (MNCtB)

RE or LE preparations of *C. mollis* were tested for genotoxicity in cells exposed for 26 hours to the extracts (0.2 μg/mL, 2 μg/mL, and 20 μg/mL) and cytochalasin B (3 μg/mL). The cells were harvested according to the protocol of Oliveira *et al.*[18] In brief, cells were trypsinized (500 μL of 0.025% trypsin-EDTA; 37°C), centrifuged (5 minutes, 215 *g*), treated with hypotonic 1% sodium citrate, and fixed (methanol:acetic acid, 3:1 vol/vol). The experiments were performed in triplicate, and 3,000 binucleated cells were examined per treatment. The criteria for the selection of the binucleated cells for the identification of micronuclei were according to Fenech,[19] and the calculation of nuclear division index was done according to Eastmond and Tucker.[20]

Test for induction of apoptosis in situ

Apoptotic cells were identified by analysis of DNA fragmentation pattern after staining with acridine orange. After stabilization in culture (24 hours), cells were treated with the concentrations of both extracts previously tested in SCGE and MNCtB, as well as 200 μg/mL. After a 24-hour treatment, cells were harvested as described by Rovozzo and Burke.[21] The cells were washed with phosphate-buffered saline, and the coverslip was removed from the culture tube and placed in Carnoy's fixative for 5 minutes. The coverslip was then immersed quickly in each container of a decreasing ethanol series (95% to 25%), followed by washing with McIlvaine's buffer for 5 minutes, staining with acridine orange (0.01%, 5 minutes), and washing again with buffer. The cells were observed under ultraviolet light. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by acridine orange. This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads.[22,23] The experiments were carried out in triplicate, and 1,500 cells were examined per treatment.

Statistical analysis

The data obtained in the MTT cytotoxicity test were subjected to analysis of variance followed by Tukey's test. Student's *t* test was used for the analysis of the data obtained for SCGE, MNCtB, nuclear division index, and apoptosis induction, where comparisons were made with the negative control. In all tests, the level of significance was set at $\alpha = 0.05$. The statistical analyses were carried out with the help of the program GraphPad (San Diego, CA, USA) Instat® version 3.02.

RESULTS

MTT cytotoxicity assay

Figures 1 and 2 show the results obtained in the MTT assay. For the RE (Fig. 1), cytotoxicity was observed beginning at 200 μg/mL. For the LE (Fig. 2) only a concentration of 2,000 μg/mL was cytotoxic to HTC cells.

Comet assay (SCGE)

We can see in Figure 3 that only exposure of cells to RE or LE at the highest concentration tested (20 μg/mL) was able to induce comets in a statistically significant manner. Cell viability remained around 90% for all treatments (Fig. 3, line).

MNCtB

The results obtained in the MNCtB revealed that there was no statistically significant increase in the number of binucleated cells with micronuclei and that there was no statistically significant alteration in nuclear division index after exposure of cells to extracts. There was only a statistically significant decrease in the number of binucleated cells with micronuclei in the cultures treated with the lowest concentration (0.2 μg/mL) of either extract (Fig. 4).

Test for induction of apoptosis in situ

Figure 5 shows that for both extracts there was a concentration-dependent increase in the number of cells undergoing apoptosis ($r = 0.966$ for RE, $r = 0.885$ for LE), and the result obtained at the highest concentration of RE and LE used (200 μg/mL) was statistically significant in relation to the negative control.

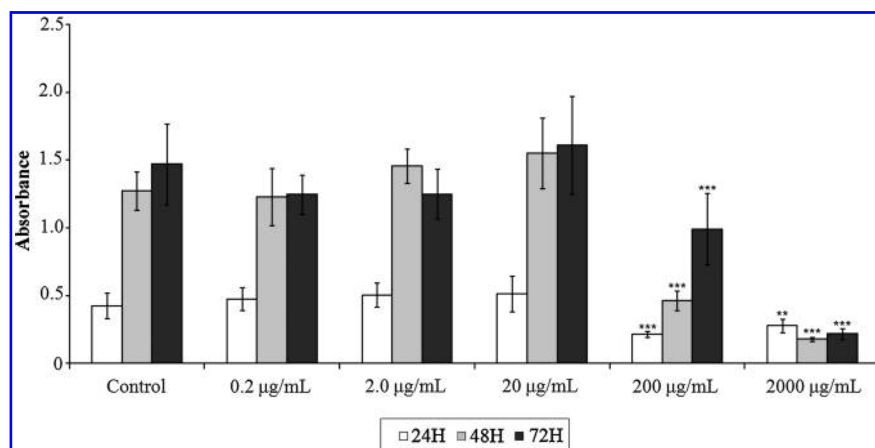

FIG. 1. Mean of the absorbance observed in the MTT assay after treatment for 24, 48, and 72 hours with ethanolic RE of *C. mollis*. Statistically significant in relation to control: $^{**}P < .01$, $^{***}P < .001$.

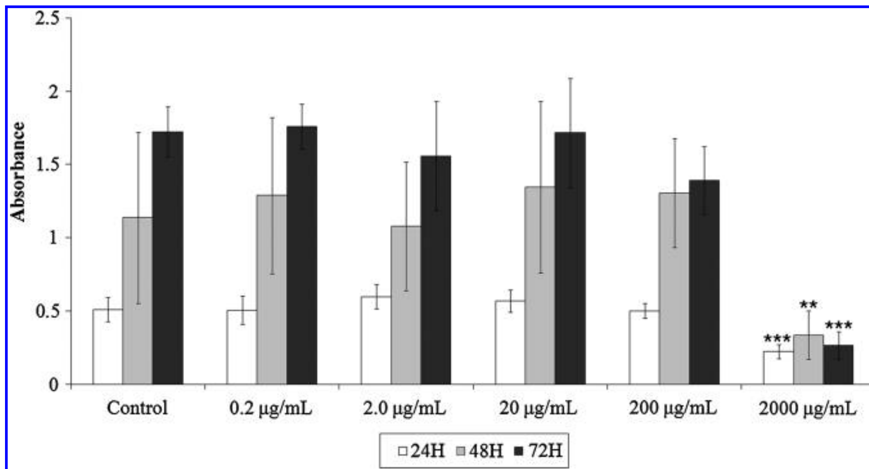

FIG. 2. Mean of the absorbance observed in the MTT assay after treatment for 24, 48, and 72 hours with ethanolic LE of *C. mollis*. Statistically significant in relation to control: $^{**}P < .01$, $^{***}P < .001$.

DISCUSSION

We observed greater cytotoxic activity of the ethanol extracts of the roots of *C. mollis* compared with the leaf extract. In RE, we identified emodin and physcion; both compounds are cytotoxic agents, and emodin has greater cytotoxic activity than physcion.[24] These compounds can be involved in the cytotoxic effect observed in the MTT assay.

Emodin and physcion are anthraquinones. This class of phytochemicals is found in various families of the plant kingdom, including the Polygonaceae family.[25] Emodin is the main active principle in various medicinal plant species and exhibits biological activities such as anti-inflammatory, antibiotic, antiviral, immunosuppressive, and hepatoprotective.[26,27] Some studies have also reported a tranquilizing activity for extracts derived from plants of the Family Polygonaceae.[28]

The results obtained with the genotoxicity tests of the extracts of *C. mollis*, under the experimental conditions described, indicate induction of DNA damage only at the highest concentration tested in SCGE and no genotoxicity in MNCtB. This nonsignificant increase in the cell number with micronuclei can be due in part to the action of the DNA repair system because the damage induced was small (only comets of Class 1).

For RE, emodin can be related to the genotoxic effect observed in the highest concentration. Mueller and coworkers attributed genotoxic activity to emodin on mammalian cells.[24,29,30] Based on studies carried out by these researchers,[24,29,30] because of its planar structure, emodin is able to intercalate double-stranded DNA and inhibit DNA topoisomerase II, resulting in DNA damages. Mutagenic response is also observed after biotransformation of emodin

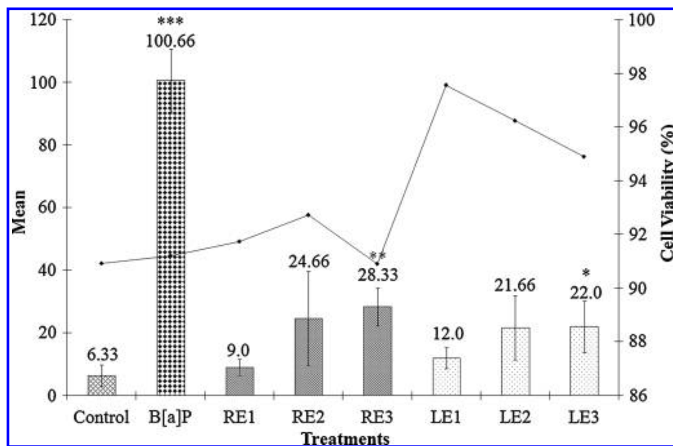

FIG. 3. Mean score of comets observed in HTC cells (columns) and cell viability (line) after a 24-hour treatment with ethanolic RE and LE of *C. mollis*: RE1, RE2, and RE3, 0.2 μg/mL, 2.0 μg/mL, and 20 μg/mL RE, respectively; LE1, LE2, and LE3, 0.2 μg/mL, 2.0 μg/mL, and 20 μg/mL LE, respectively. B[a]P, benzo[*a*]pyrene (25 μg/mL). Data are mean values of three cultures; 100 cells were analyzed in each culture for genotoxicity. $^{*}P = .0382$, $^{**}P = .0057$, $^{***}P = .0001$.

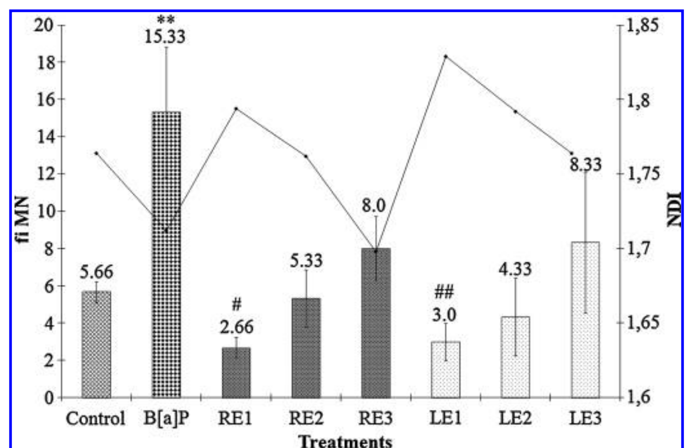

FIG. 4. Mean of binucleated cells with micronucleus (MN) (columns) and nuclear division index (NDI) (line) after a 26-hour treatment with ethanolic RE and LE of *C. mollis*: RE1, RE2, and RE3, 0.2 μg/mL, 2.0 μg/mL, and 20 μg/mL RE, respectively; LE1, LE2, and LE3, 0.2 μg/mL, 2.0 μg/mL, and 20 μg/mL LE, respectively. B[a]P, benzo[*a*]pyrene (25 μg/mL). Data are mean values of three cultures; 1,000 cells were analyzed in each culture for mutagenicity, and 500 were cells analyzed in each culture for NDI. $^{\#}P = .0031$, $^{\#\#}P = .0161$, $^{**}P = .0093$.

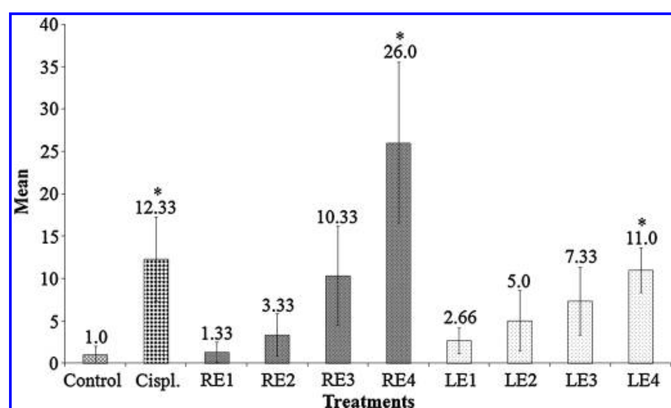

FIG. 5. Mean of apoptotic cells observed after a 24-hour treatment with ethanolic RE and LE of *C. mollis*: RE1, RE2, RE3, and RE4, 0.2 μg/mL, 2.0 μg/mL, 20 μg/mL, and 200 μg/mL RE, respectively; LE1, LE2, LE3, and LE4, 0.2 μg/mL, 2.0 μg/mL, 20 μg/mL, and 200 μg/mL LE, respectively. Cispl., cisplatin (6 μg/mL). Data are mean of three cultures; 500 cells were analyzed in each culture. *P (Cispl.) = .0175, *P (RE4) = .0107, *P (LE4) = .0036.

via cytochrome P 450 1A2 and 2B, which produces more mutagenic metabolites such as 2-hydroxyemodin and therefore probably is generated by HTC cells (proficient in drug metabolism).

In relation to the experiments with LE, it is difficult to state or even suggest the reason behind the genotoxic result because little is known about the long-chain hydrocarbons, carboxyl esters, and 3-taraxerone observed in this extract.

Both extracts were also able to induce apoptosis. For RE, emodin can also be involved, as it showed the ability to induce apoptosis.[31–34] According to Olsen *et al.*,[35] this ability can be the main mechanism of the antiproliferative effect in tumor cells when exposed to emodin.

For LE, the triterpene 3-taraxerone identified can contribute to the cytotoxicity and apoptosis induction found with HTC (rat hepatoma cells) because it is known that other compounds of this class (ursolic and oleanolic acids) can induce this process in different cell lines, including HepG2 (human hepatoma cells).[36–38] Also, a modified form of 3-taraxerone (3,4-*seco*-taraxerone) has shown cytotoxicity in the same human cell.[39]

The experimental results obtained in our study demonstrate that RE shows a greater toxicity than LE, probably because of the presence of emodin. The data from the apoptosis assay indicate that the reduction in viable cells found in the MTT assay is partly the result of the induction of apoptosis in the HTC cells. The results of the highest concentration tested of RE (20 μg/mL) in MNCtB and in the test for induction of apoptosis *in situ*, despite not being statistically significant, can also suggest that a greater induction of DNA damage could have occurred than that detected by the *in situ* test because nuclear DNA fragmentation is a late event in the process of apoptosis,[40] but without compromise of the formation of micronuclei (nuclear division index not significant).

The results presented here for the first time, along with a better understanding of the biological activities of the extracts of *C. mollis*, are important for scientifically confirming the popularly proposed effects and for providing greater security to people using this medicinal plant.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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