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Evaluation of genotoxic and cytotoxic effects of hydroalcoholic extract of *Euphorbia tirucalli* (Euphorbiaceae) in cell cultures of human leukocytes

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ABSTRACT

*Euphorbia tirucalli* (L.), commonly known as aveloz, has been indiscriminately used in popular medicine to treat various illnesses. However, some components can have devastating consequences. Injury to a cell’s genetic material can cause mutations, cancer, and cell death. Our main goal in this work was to evaluate the genotoxic and cytotoxic effects of *E. tirucalli* extract on human leukocytes. For this purpose, we performed a phytochemical analysis to evaluate the plant’s components. In the second step, we treated cultured human leukocytes with different concentrations of the dry extract of the plant and then evaluated the oxidative and genotoxic profiles of these leukocytes. We found that at 1% and 10% concentrations, the aveloz extract acted as a genotoxic agent that could damage DNA and increase oxidative damage. We conclude that despite its popular use, aveloz can act as a genotoxic agent, especially when it contains phorbol ester. Aveloz’s indiscriminate use might actually promote tumors and therefore carry a considerable genetic risk for its users.

Key words: Aveloz, *Euphorbia tirucalli*, leukocytes, genotoxic, cytotoxic.

INTRODUCTION

Approximately three-quarters of the world’s population use plants as a source for substances with pharmacological and therapeutic properties primarily to treat diseases and maintain health. Therefore, a significant number of people directly depend on alternative medicine as a primary form of treatment for chronic diseases (Mengue et al. 2001, Ritter et al. 2002, Mendonça Filho and Menezes 2003, Pereira et al. 2004, Vendruscolo et al. 2005, Carlini 2003, Agra et al. 2007, Biavatti et al. 2007).

*Euphorbia tirucalli* (L.), popularly known as pencil tree, dog’s stick, or aveloz, is a plant indigenous to Africa but well adapted in Brazil that has been indiscriminately used in popular medicine to treat various illnesses and conditions, including snake bites, asthma, and muscle spasms (Valadares et al. 2006). Moreover, aveloz has been reported...
to have antiviral and antimicrobial properties (Jurberg et al. 1985), as well as molluscicidal and larvicidal properties (Yadav et al. 2002). Aveloz also has been used as a home treatment for various types of cancer (Madureira et al. 2004). This plant presents a wide range of bioactive constituents, including quercetin, rutin, gallic acid, caffeic acid, taraxasterol, tirucallol, 12-O-tetradecanoylphorbol-13-acetate (TPA), ingenane, togliane, and diterpenic acid derivatives (Fürstenberger and Hecker 1986). Interestingly, some reports have indicated that the pharmacological properties of the crude plant extracts can be lost after specific compounds are isolated; this indicates that at least some of their pharmacological properties may result from the combination of different classes of compounds (Pietrovski et al. 2006, Carlini 2003).

Reactive oxygen species (ROS) are generated by normal metabolic processes in all organisms that use oxygen (Frei 1994, Silva et al. 2005, Finkel and Holbrook 2000). However, excessive ROS production can overcome cellular antioxidant defenses and can lead to oxidative stress. Oxidative stress is thought to contribute to the development and progression of several degenerative diseases via DNA mutation, protein oxidation or lipid peroxidation (Finkel and Holbrook 2000, Valko et al. 2004).

Medicinal plants have been traditionally used to treat several human diseases, and their pharmacological and therapeutic properties have been attributed to different chemical constituents isolated from their crude extracts. Of particular importance, chemical constituents with antioxidant activity can be found in plants at high concentrations and are likely to be responsible for their protective effects against various degenerative diseases, including cancer, neurological, and cardiovascular diseases (Evans et al. 2006, Mentreddy 2007, Leite et al. 1986, Velioglu et al. 1998). Thus, plants have many prospective applications in human healthcare because of their antioxidant properties (Silva et al. 2005).

Despite the potential benefits of polyphenols, some products derived from plants can contain toxic compounds. For example, *E. tirucalli* contains TPA, a dangerous phorbol ester (Lin et al. 2012). Thus, indiscriminate use of crude plant products can have devastating consequences, and these consequences are not always immediately apparent. Injury to a cell’s genetic material can cause mutations, cancer, and cell death within a very point in post-exposure period (da Silva et al. 2011). Genotoxic studies have been conducted to evaluate such risks (Kumar et al. 2010). Our main goal in this work was to evaluate the genotoxic and cytotoxic effects of *E. tirucalli* extract on human leukocytes, the primary barrier of defense for the human immune system.

**MATERIALS AND METHODS**

**CHEMICALS**

All chemicals used in this work were of analytical grade. Solvents for the extracts and *L*-Ascorbic acid were purchased from Merck (Darmstadt, Germany). All other reagents were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

**PLANT MATERIAL**

The aerial parts of *E. tirucalli* were harvested in March 2008 at Bagé (31°19′51″S/54°6′25″W) (State of Rio Grande do Sul, Brazil). Samples of the collected material were identified by the botanist, Dr. Thais Scott do Canto Dorow, and archived as voucher specimens by register number SMD 10127 in the herbarium of the Department of Biology at Federal University of Santa Maria. Extracts were obtained by adding the fresh aerial parts of *E. tirucalli* to ethanol (70%) and storing the mixture at room temperature for seven days with daily agitation. After seven days, the mixture was filtered and the extract was evaporated under reduced pressure to remove the ethanol, which resulted in a crude extract yield of 6.82%.
DETERMINATION OF TOTAL POLYPHENOLIC CONTENTS

The total polyphenol concentration in the crude extract was measured spectrophotometrically using a modified Folin-Ciocalteau method (Chandra and Gonzalez de Mejia 2004). Briefly, we added 0.5 mL of 2 N Folin-Ciocalteau reagent to 1 mL of each sample (0.15 mg/mL) and allowed this mixture to stand for 5 min before adding 2 mL of 20% Na₂CO₃. Next we allowed the solution to stand for 10 min before reading it in a Shimadzu-UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan) at 730 nm. We estimated the phenolic compounds in the crude extract in triplicate. Because of the chemical heterogeneity of plant products and the specificity of phenolic reagents, it is very difficult to choose suitable standards for identifying all phenols in a plant extract. Aqueous solutions of gallic acid in the range of 0.001-0.2 mg/mL as references was used. Thus, it is only possible to get relative equivalents with the standard used. The total polyphenol content was expressed as milligram equivalents of gallic acid per milliliter of the extract.

DETERMINATION OF CONDENSED TANNINS

Methanolic solutions of rutin in the range of 0.001-0.2 mg/mL as references was used. The crude extract was prepared with a standardized procedure of dissolving 0.25 g in 10 mL of methanol. The final concentration was 25 mg/mL. The total condensed tannin concentrations in crude extract was measured spectrophotometrically using the modified vanillin method (Morrison et al. 1995). Briefly, we added 0.9 mL of methanol to 0.1 mL of each sample (25 mg/mL). To this mixture, we added 2.5 mL of Solution A (8 mL of HCl in 100 mL of methanol) and 2.5 mL of Solution B (1 g of vanillin in 100 mL of methanol). We heated the solution for 10 min at 60 °C before reading it in the spectrophotometer at 730 nm. The blank was prepared with 0.1 mL of water in 0.9 mL of methanol, 2.5 mL of Solution A, and 2.5 mL of Solution B. We determined the condensed tannins in the crude extract in triplicate. The contents were expressed as milligram equivalents of rutin per milliliter of the extracts.

DETERMINATION OF TOTAL FLAVONOIDS

Methanolic solutions of quercetin in the range of 4.0–12.0 μg/mL as references was used. To 2 mL of each reference solution, we added 20 mL of methanol and 1 mL of 5% methanolic solution of AlCl₃ (w/v) and produced a total volume of 50 mL with methanol at 20 °C. After 30 min, we measured the absorbance in a Shimadzu-UV-1201 spectrophotometer at 425 nm. We used the same procedure to analyze the crude extract. The blank was 5% AlCl₃ (w/v) (Woisky and Salatino 1998). We estimated the flavonoids in crude extract in triplicate. The contents were expressed as milligram equivalents of quercetin per millilitre of the extracts.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

**Apparatus:** The extracts were examined with high performance liquid chromatography (HPLC) using an HPLC system (Shimadzu, Kyoto, Japan), Prominence auto sampler (SIL-20A), which was equipped with Shimadzu LC-20 AT reciprocating pumps connected to a degasser DGU 20A5 with an integrator CBM 20A, a UV-VIS detector DAD (diode) SPD-M20A, and the Software LC solution 1.22 SP1.

**Analysis of phenolic compounds:** Reverse phase chromatographic analyses were performed under gradient conditions using a C18 column (4.6 mm × 150 mm) packed with 5-μm diameter particles. The mobile phase was water containing 2% acetic acid (A) and methanol (B). The composition gradient was 5% of B to 2 min, when it was changed to obtain 25%, 40%, 50%, 60%, 70%, and 100% B at 10, 20, 30, 40, 50, and 80 min, respectively, following
the method described by Laghari and colleagues, with slight modifications (Laghari et al. 2011). The extracts were analyzed at a concentration of 0.150 mg/mL. We investigated the presence of the following six antioxidants: gallic acid, chlorogenic acid, caffeic acid, quercetin, rutin, and kaempferol. These compounds were identified by comparing their retention times and UV absorption spectra with those of the commercial standards. The flow rate was 0.7 mL/min and the injection volume was 40 μL. The wavelengths were 254 nm for gallic acid; 327 nm for caffeic and chlorogenic acids; and 365 nm for quercetin, rutin, and kaempferol. The samples and mobile phase were filtered through a 0.45-μm membrane filter (Millipore) and then degassed in an ultrasonic bath before use. Stock solutions of reference standards were prepared in the HPLC mobile phase for quercetin, rutin, and kaempferol at 0.020–0.200 mg/mL and for gallic, caffeic, and chlorogenic acids at 0.050–0.250 mg/mL. A chromatography peak was confirmed by DAD spectra (200–500 nm) and by comparing its retention time with that of a reference standard.

Analysis of 12-O-tetradecanoylphorbol-13-acetate (TPA): TPA was identified using a gradient elution HPLC method described by Makkar and colleagues (Makkar et al. 1997). Briefly, samples were injected into a C18 column (4.6 mm × 250 mm) packed with 5-μm diameter particles. The samples initially contained 60% of solvent A (1.75 mL of 85% o-phosphoric acid in 1 L water) and 40% of solvent B (acetonitrile), and B was increased to 50% over 10 min, 75% from 10 to 30 min, and 100% from 30 to 45 min. The column was then washed with solvent C (2% tetrahydrofuran in methanol). Detection occurred at 280 nm. Stock solutions of reference standards was prepared in the HPLC mobile phase at 0.020–0.200 mg/mL. A chromatography peak was confirmed by DAD spectra (200–500 nm) and by comparing its retention time with that of a reference standard.

HUMAN BLOOD SAMPLES

Peripheral blood was collected by venipuncture into sterile vials containing 68 I.U. of sodium heparin (BD Vacutainer®) per mL of blood. The vials were transferred to the laboratory, and whole-blood cultures were established. The blood samples were stored up to 24 h at 4 °C before culturing. This project was approved by the University’s Committee of Ethics in Research of Universidade Federal de Santa Maria (RS) (authorization nº 23081.012330/2006-94).

CULTURE CELL PREPARATION

The lymphocyte cultures were prepared with whole-blood samples and immediately transferred to 1 mL of culture medium containing RPMI 1640 supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin, as previously described (dos Santos Montagner et al. 2010). The cells were then placed in a microaerophilic environment at 37 °C for 72 h. The solutions under investigation were added with the blood with a 10% concentration. The solutions analyzed included crude extract of *E. tirucalli* L. diluted in phosphate-buffered saline (PBS) at concentrations of 0.001%, 0.01%, 0.1%, 1%, and 10%. TPA, the primary active component in *E. tirucalli* L., was used as a positive control and at the same concentration found in the plant’s phytochemical analysis. Each group consisted of three culture flasks. Genetic and oxidative parameters were analyzed after 72 h of growth.

ANALYSIS OF OXIDATIVE PARAMETERS

Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Ohkawa et al. 1979, dos Santos Montagner et al. 2010). Superoxide dismutase (SOD) (E.C.1.15.1.1) activity was measured spectrophotometrically according to the procedure described by Boveris and Cadenas (1997). The unit of activity was defined as the
amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50%. Catalase activity (EC 1.11.1.6.) was determined according to the procedures described in a previous report (Aebi 1984). One unit of catalase activity was defined as the activity required to degrade one mol of hydrogen peroxide within 60 s. The protein carbonyl was also quantified according to previously described procedures (Morabito et al. 2004). Total Protein content was determined using the commercially available kit of Wiener Lab. (Rosario, Argentina), kindly donated by the company. The results were expressed in nanomoles of carbonyl groups per mg of protein.

**ANALYSIS OF GENOTOXIC PARAMETERS**

To perform the genotoxicity tests, we first counted the total number of leukocytes in a Neubauer chamber (dos Santos Montagner et al. 2010). Viability was assessed by a loss of membrane integrity, which was indicated with trypan blue (Burow et al. 1998). Overall, we counted 300 cells. The genotoxicity test was conducted using comet assay (Singh et al. 1988). Although comet assay is not the only method for measuring oxidative DNA damage, it is one of the most sensitive and accurate and is relatively free of artifacts (Collins 2009). We identified 100 cells in the slides that were submitted for analysis. The cells were visually scored according to tail length, with scores ranging from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The tests were carried out in triplicate, and the data are presented as mean ± standard error.

The mitotic index and chromosomal instability test were carried out using Cytogenetics Band G. Fifty mitoses were analyzed per sample (Yúnis 1976, dos Santos Montagner et al. 2010). In the micronucleus test (MN), the cells were fixed with acetic acid and methanol (75:25, v/v), transferred onto clean microscope slides in duplicates, and then stained with 5% Giemsa. The criteria for scoring cells with MN were described in a previous report (Thomas et al. 2008). One thousand cells were counted for each sample, and the results were expressed as the micronucleus frequency per 1000 cells.

**STATISTICAL ANALYSIS**

Statistical software was used to perform all statistical analyses, which included an analysis of variance (ANOVA) followed by a post hoc Bonferroni test. P values < 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites found in plants (Komali et al. 1999). Flavonoids occur naturally in plant foods and are a common component of our diet. They generally occur as O-glycosides with sugars bound at the C3 position. Flavonoids have a wide range of biochemical and pharmacological effects, including antioxidant, anti-inflammatory, and antifungal effects (Komali et al. 1999). Analysis of condensed tannins is complicated by the diversity of structures found within this group of compounds. The vanillin method depends on the reaction between vanillin and condensed tannins and the formation of colored complexes (Muchuweti et al. 2006). Tannins have been reported to exert other physiological effects, such as reducing blood pressure, accelerating blood clotting, decreasing serum lipid levels, modulating immune responses, and causing liver necrosis (Zu et al. 2006). **Table I** shows the concentrations of these metabolites and some of their constituent compounds, all of which show biological activity. The HPLC-DAD method is most commonly used to identify phenolic compounds (Paganga et al. 1999). **Table I** also presents the concentrations of seven biologically active compounds.
The samples were prepared from the extracts indicated in Table I. Table II shows the final concentrations of these markers produced with the treatments used in this protocol.

Figures 1, 2, and 3 demonstrate that treating cultured human leukocytes with varying concentrations of *E. tirucalli* affected their biological parameters. Regarding the proliferation of leukocytes, the only treatments that caused interference were those with TPA (increase of 50 ± 6.9%) and extract at 10%. These treatments showed an increase in proliferation at 17% ± 2.8%. TPA has been described extensively in the literature, which includes reports of proliferative activity (Xu et al. 2015, Shi et al. 2013). The proliferation observed with 10% extract was consistent with this characteristic, as Table II shows moderate concentrations of 12-O-tetradecanoylphorbol-13-acetate. This same effect is also illustrated in Figure 1c. When evaluating the mitotic index, we observed that samples containing higher amounts of TPA had higher rates of cell multiplication, as demonstrated by a cell’s increase in mitotic indices. Figure 1b indicates the cell viability means ranged from 0.33 ± 0.57% to 6.0 ± 1.0%. The effects of TPA also were reflected in these samples by increased cell death rates. The phorbol esters can induce inflammatory responses (Wei et al. 2011) and NF-kB expression, which signals a cascade of events leading to apoptosis and a subsequent reduction of leukocytes in culture (Nomura et al. 2000).

### Table I

Concentrations of analytical markers present in the crude extract of *Euphorbia tirucalli* L.

| Group / Compound       | Concentration in the crude extract (μg/mL) | Standard Curve | R²   |
|------------------------|--------------------------------------------|----------------|------|
| Polyphenol compounds¹  | 2072.00 ± 4.07 y = 34.443x – 0.0942         | 0.9937         |
| Condensed tannins¹     | 689.50 ± 24.64 y = 0.0423x + 0.1362         | 0.9849         |
| Total flavonoids¹      | 882.00 ± 41.61 y = 0.0202x + 0.0031         | 0.9999         |
| Quercetin²             | 1.47 ± 0.11 y = 32214x – 259717              | 0.9968         |
| Rutin²                 | 0.49 ± 0.07 y = 19217x – 16913               | 0.9998         |
| Gallic acid²           | 30.52 ± 1.19 y = 7606.8x – 132936            | 0.9703         |
| Caffeic acid²          | 15.61 ± 3.12 y = 20367x – 1162400            | 0.9890         |
| Chlorogenic acid²      | 12.37 ± 2.69 y = 12158x + 1174.9             | 0.9996         |
| Kaempferol²            | 3.45 ± 0.11 y = 12647x + 1178.3              | 0.9995         |
| 12-O-tetradecanoylphorbol-13-acetate (TPA)² | 3.12 ± 2.87 y = 27228x – 31278           | 0.9971         |

¹Determined by spectrophotometric techniques. ² Determined by HPLC-DAD. Data are expressed as mean ± S.D (n=3).

### Table II

Concentrations of bioactive compounds identified in samples according to treatment.

| Compound                       | Concentrations of compounds in each treatment used in this protocol (μg/mL) |
|--------------------------------|--------------------------------------------------------------------------------|
|                                | PBS (NC)           | TPA (PC)           | 0.001% | 0.01% | 0.1% | 1% | 10% |
| Quercetin                      | Nd                 | Nd                 | Nd     | Nd    | Nd   | Nd | 0.15 |
| Rutin                          | Nd                 | Nd                 | Nd     | Nd    | Nd   | Nd | 0.05 |
| Gallic acid                    | Nd                 | Nd                 | Nd     | 0.03  | 0.31 | 3.05 |
| Caffeic acid                   | Nd                 | Nd                 | Nd     | 0.02  | 0.16 | 1.56 |
| Chlorogenic acid               | Nd                 | Nd                 | Nd     | Nd    | 0.12 | 1.24 |
| Kaempferol                     | Nd                 | Nd                 | Nd     | Nd    | 0.03 | 0.35 |
| 12-O-tetradecanoylphorbol-13-acetate (TPA) | Nd               | 3.12               | Nd     | Nd    | Nd   | 0.03 | 0.31 |

The data are presented in mean values (n=3), based on the results presented in Table I. Nd: not detectable.
Figure 2 presents the findings of the oxidative parameter analysis. In Figure 2a and b, we see the results for two major targets of free radicals, lipids and proteins. Figure 2c and 2d describe the activity of two key antioxidant enzymes, SOD and catalase.

The TBARS is a classical model for lipid peroxidation analyses (Ohkawa et al. 1979). Figure 2a reports the levels of lipid peroxidation upon exposure to varying concentrations of *E. tirucalli* extract. As the figure indicates, the only treatment that increased lipid peroxidation levels was the control TPA (18.11 ± 3.73%). Concentrations of 0.1–10% showed reduced levels and reached a reduction of 68.9 ± 3.73% at the highest concentration. Figure 2b shows that the protein carbonyl content also increased (107.00 ± 23.89%), but only with TPA. Unlike lipid peroxidation, groups with the extract showed no statistically significant changes. In Figure 2c and d, the antioxidant enzyme activity of SOD and catalase showed no significant changes with any treatment. The compound 12-O-tetradecanoylphorbol-13-acetate can induce reactive species production; therefore, we expected an increase in lipid peroxidation and protein carbonylation parameters (Khan et al. 2013). Plant extracts are sources of several potentially beneficial compounds, including the phenolic compounds tested here. These results confirm previous findings that show phenolic compounds, especially flavonoids, participate in oxidative metabolism by directly neutralizing the reactive species (Liu et al. 2008).

Figure 1 - Markers of cell proliferation. The bar graphs indicate total leukocytes counted (a), percentage of cell inviability (b), and mitotic index (c). PBS: phosphate-buffered saline, TPA: 12-O-tetradecanoylphorbol-13-acetate, EtCE: *Euphorbia tirucalli* crude extract. Data are presented as mean ± SD (n = 3). Letters indicate statistically significant differences.
Figure 3 illustrates the treatment effects for the genetic evaluation in toxicity. Specifically, Figure 3 illustrates the frequency of micronuclei (a), DNA damage by comet assay (b), and chromosomal abnormalities (c).

A similar pattern is apparent in the test results illustrated in Figure 3a-c. In all cases, TPA was introduced as a genotoxic agent, which subsequently increased the micronuclei (about five times), DNA damage (approximately 40 times), and the number of chromosomal aberrations (about 68 times). Such effects are widely reported and well characterized in previous studies (Okabe et al. 2011, Kumar et al. 2012, Kawabe et al. 2013).

Measurements of the micronucleus frequency in cultured human lymphocytes were used to evaluate chromosomal damage following exposure to genotoxic agents (Nefic and Handzic 2013). We observed an increased frequency of micronuclei, thus indicating a genotoxic risk at 1% and 10% concentrations of crude extract, with increases ranging from three to five times that of the negative control (PBS). The same observations were made when the frequency of chromosomal aberrations was evaluated by determining the numerical and structural chromosomal abnormalities. The analysis also indicated a genotoxic risk with concentrations of 1% and 10%, with chromosome damage...
increasing from 44 to 68 times, respectively. The comet assay, also called the single cell electrophoresis assay, can determine DNA strand breaks after in vitro exposures. The comet assay involves electrophoresis of lysed cells embedded in agarose on a microscope slide. Undamaged DNA remains within the core, whereas fragmented DNA migrates from the core toward the anode, forming an image that resembles a comet with a head and tail (Lee et al. 2013). We identified genotoxic damage only in cultures containing the crude extract at 10%, indicating an increase in DNA breakage that was 38 times higher than that for the negative control resembling the treatment with TPA. As Table II indicates, we found that extract samples at 1% and 10% contained higher contents of active compounds, which included polyphenols, phorbol ester, and TPA. Our findings demonstrate that these extracts have high genotoxic potential. Polyphenols have been extensively reported as having antigenotoxic activity and highly protective effects for genetic material (Zheng et al. 2010, Rajavelu et al. 2011, Katiyar 2011, Pedret et al. 2012). On the other hand, TPA is a genotoxic agent (Okabe et al. 2011, Kumar et al. 2012, Kawabe et al. 2013). We therefore conclude that the

Figure 3 - Markers of genotoxic effects. Markers were the frequency of micronuclei (a), DNA damage by comet assay (b), and chromosomal abnormalities (c). PBS: Phosphate-buffered saline, TPA: 12-O-tetradecanoylphorbol-13-acetate, EtCE: Euphorbia tirucalli crude extract. Data are presented as mean ± SD (n = 3). The letters indicate statistically significant differences.
present findings reflect TPA’s effects on the plant composition, and therefore, the extract used in the tests.

Even though this plant is popularly used to treat various diseases, it is potentially a genotoxic agent, especially in the presence of phorbol ester. Its indiscriminate use can actually promote tumors and confer large genetic risks to its users.

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