

Original Research Article

Antiproliferative activity of extracts of *Euphorbia tirucalli* L (Euphorbiaceae) from three regions of Brazil

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Abstract

Purpose: To investigate *Euphorbia tirucalli* extract for probable geographic variations in its antiproliferative activity.

Methods: The aerial parts of *E. tirucalli* were collected in the Brazilian states of Mato Grosso, Rio de Janeiro, Pará, Minas Gerais and Santa Catarina. The 70 % ethanol extract was obtained according to the procedure described in Brazilian Homeopathic Pharmacopeia. The antiproliferative activity of extracts, in concentrations of 62, 125, 250, and 500 $\mu\text{g mL}^{-1}$, was tested against leukemia (HL-60), lymphoma (Daudi) and melanoma (B16F10) cell lines using methyl thiazol tetrazolium assay (MTT). Phytochemical analysis were carried out using High-performance liquid chromatography-diode array (HPLC-UV-DAD) and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI(-) FT-ICR MS) assays.

Results: There was significant regional variability in the cytotoxicity of *E. tirucalli* extracts in a dose-dependent manner. The extracts had similar activity towards leukemia cell line HL-60, decreasing cell viability to about 60 – 70 %. The extract showed the presence of ellagitannins, flavonoids, veracylglycan, and acid triterpenes as the major compounds.

Conclusion: While the results support the ethnopharmacological use of *E. tirucalli* throughout Brazil, regional quantitative differences found in some classes of secondary metabolites may explain the variations observed in antitumor activity.

Keywords: Aveloz, Cancer, Cytotoxicity, Antiproliferative, Ethnopharmacological, Traditional medicine

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INTRODUCTION

Euphorbia tirucalli L. (Euphorbiaceae), commonly known as aveloz or pencil cactus, is a shrub of wide occurrence in Africa and Brazil. The popular use of *E. tirucalli* in Africa has been associated with a high incidence of Burkitt's lymphoma [1]. However, several studies have also shown the

non-toxicity of aveloz and, as such, it is used for the treatment of cancer in some countries. The wide use of *E. tirucalli* as a medicinal aid is found among Brazilian indigenous communities to treat rheumatism, stomach ailments, and asthma [2]. In Brazil, as well as in other tropical and subtropical countries, *E. tirucalli* is also commonly used in folk medicine for the treatment

of cancer [3-5]. For instance, it is still recommended by Brazilian Homeopathic Pharmacopeia [6] and widely used in popular medicine. Specifically, a prescription of six drops of *E. tirucalli* latex diluted in 2 liters of water was indicated to treat neoplasm [7]. As also recommended by Brazilian Pharmacopeia, alcoholic solutions obtained from fresh plant material (syrops or tinctures) have been used for medicinal purposes. Since 1997, the use of ultra-diluted extracts of *E. tirucalli* against cancer and AIDS has been investigated in homeopathic clinics [8]. Studies have shown that high dilutions of *E. tirucalli* latex modify the viability and glycolytic metabolism of nontumor melanocytes and human breast cancer cells [9].

Interestingly, Brazil is a country that presents a wide variety of geographic regions and climatic conditions that might influence the production of secondary metabolites, resulting in potential variation in the potency of this plant's anticancer activity. Nevertheless, *E. tirucalli* is popularly used in uniform dosages throughout the country. Therefore, to determine the antiproliferative activity of *E. tirucalli* plants collected in different areas of Brazil, three cancer cell lines were tested and analyzed by MTT, and tinctures were chemically analyzed. Diterpenes, triterpenes, steroids, flavonoids and ellagitannins have been described to occur in *Euphorbia tirucalli* samples from different parts of the world [10-12].

EXPERIMENTAL

Collection of plant materials

Stems of *E. tirucalli* were collected from August to December, 2005, in different cities in Brazil (10° S, 55° W, South America): Cáceres (Mato Grosso, MT), Rio de Janeiro/UFRJ (Rio de Janeiro, RJ), Arraial do Cabo (Rio de Janeiro,

RJ), Belém (Pará, PA), Montes Claros (Minas Gerais, MG) and Araranguá (Santa Catarina, SC). These areas were chosen on the basis of different climatological and ecological conditions and the representative popular use of this plant.

Table 1 shows the latitude and longitude and climatic features of each location based on data obtained from the National Institute for Meteorology (INMET, Brazil), as well as Köppen climate classification scheme. All specimens of *E. tirucalli* were identified by Luci de Senna Vale (National Museum, Federal University of Rio de Janeiro), and they were deposited in the Biology Institute Herbarium, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil (Table 1).

Preparation of extracts

E. tirucalli stems (aerial parts) were collected from 11 to 12h. Three samples (extracts) were prepared from fresh plant for each municipality. Twenty grams of plant material were immersed in 100 mL of 70 % ethanol and allowed to macerate in a shaker for a week according to the procedure described in the Brazilian Homeopathic Pharmacopeia [6].

Crude hydroalcoholic extracts were filtered and dried through evaporation at 60 °C in a rotatory evaporator, followed by freeze-drying. For antiproliferative assays, the freeze-dried material was dissolved in dimethyl sulfoxide (DMSO 99.9 %, Sigma, St. Louis, MO) and then diluted in culture medium. The extracts were stored at -20 °C until use.

High-performance liquid chromatography (HPLC)

HPLC-UV-DAD analyses were performed in a Shimadzu apparatus equipped with a SPD-M10A

Table 1: Summary of geographic/climatic characteristics of each city where *Euphorbia tirucalli* was collected and herbarium voucher no.

Location in Brazil (Municipality)	Latitude/longitude	Average temperature (°C)	Average precipitation (mm)	Climate*	Voucher no.
		Aug.-Dec.2005	Aug.-Dec.2005		
Montes Claros (MG)	16° 44' 06"S 43° 51' 43"W	24.2	140	Aw	RFA-31677
Belém (PA)	01° 27' 21"S 48° 30' 14"W	27.0	272	Af	RFA-31676
Cáceres (MT)	16°04' 14"S 57°40' 44"W	20.1	78	Aw	RFA-31674
Araranguá (SC)	28° 56' 06" S 49° 29' 09"W	24.0	120	Cfa	RFA-31679
Arraial do Cabo (RJ)	22° 57' 57"S 42° 01' 40"W	20.0	145	Af	RFA-31678
Rio de Janeiro/UFRJ (RJ)	22° 56' 26"S 43° 28' 50"W	23.5	118	Af	RFA-31675

diode array detector, LC-10AD pump and CBM-10 interface. For analysis, 10 mg of samples were diluted in 1 mL of absolute ethanol, and 20 μL of this solution were injected. DAD-HPLC analyses were run in a reverse phase column (LiChrosorb RP-18, 25 cm x 5 mm) at ambient temperature. Separation was done in the following mobile phase: type 1 ultrapure water (A) and methanol (B) with gradient of 50 to 90 % B, in 40 minutes, with flow rate of 1 mL min^{-1} . A photodiode detector focused at 240 and 360 nm was used. Flavonoid and tannin chemical classes were detected through retention times, and UV spectral characteristics were compared with standard flavonoid and tannin spectra and previous studies reporting on the phytochemistry of *E. tirucalli* [12,13]. All chemicals used in the analysis were of HPLC grade and were purchased from Merck. Analyses were performed in triplicate.

ESI(-) FT-ICR MS

The chemical profile of ethanolic extracts of *E. tirucalli* (10 μL) was explored using negative-ion electrospray ionization Fourier transform ion cyclotron mass spectrometry (ESI(-) FT-ICR MS). Briefly, the samples were diluted in water:acetonitrile (1:1) which contained 0.1 % m/v of NH_4OH . The resulting solution was directly infused at a flow rate of 5 $\mu\text{L min}^{-1}$ into the ESI source. The mass spectrometer (model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany) was set to operate in negative ion mode over a mass range of m/z 200 - 2000. ESI source conditions were as follows: nebulizer gas pressure of 0.5 - 1.0 bar, capillary voltage of 3.0 - 3.5 kV, and transfer capillary temperature of 250 $^{\circ}\text{C}$. Mass spectra were acquired and processed using the Compass Data Analysis software package (Bruker Daltonics, Bremen, Germany). A resolving power, $m/\Delta m_{50\%} \cong 500\,000$, in which $\Delta m_{50\%}$ is the full peak width at half-maximum peak height of $m/z \cong 400$ and a mass accuracy of < 1 ppm, provided the unambiguous molecular formula assignments for singly charged molecular ions. Elemental compositions of the compounds were determined by measuring the m/z values. The aromaticity of each molecule was directly deduced from its DBE value according to Equation 1:

$$\text{DBE} = c - h/2 + n/2 + 1 \dots\dots\dots (1)$$

where c, h, and n are the numbers of carbon, hydrogen, and nitrogen atoms, respectively, in the molecular formula.

Cell lines

Cancer cell lines B16F10 (murine melanoma), HL - 60 (promyelocytic leukemia) and Daudi (Burkitt's lymphoma) (from the National Cancer Institute, Bethesda, MD, USA) were used. Cell lines were cultivated in DMEM (B16F10) or RPMI (HL - 60 and Daudi) supplemented with 10 % FBS, 100 $\mu\text{g mL}^{-1}$ of penicillin, 100 $\mu\text{g mL}^{-1}$ of streptomycin, 2 mM glutamine, and 0.07 % NaHCO_3 . The cultures were maintained at 37 $^{\circ}\text{C}$ in a humidified 5 % CO_2 atmosphere.

Cell viability assay

Cell viability was assessed by MTT (methyl thiazol tetrazolium). After 24 h resting, plated cells ($2 \times 10^4/\text{well}$) were treated with medium, the desired concentrations of extracts (62, 125, 250, and 500 $\mu\text{g mL}^{-1}$), or DMSO at the same concentrations carried by the material, and incubated for another 48 h. After incubation, each well received 2.5 mg/mL of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide], and the plates were incubated for an additional 4 h at 37 $^{\circ}\text{C}$. The medium was removed, and the crystals of reduced formazan were dissolved with 150 μL of DMSO. Positive control, cisplatin, was also used against these cell lines. Absorbance was determined at 570 nm with a microplate reader (BenchMark, Bio-Rad, Hercules, CA). Effects of the extract on cell viability were calculated, using cells treated with DMSO as control. At the higher concentration used (1 %), DMSO had no effect on cell viability. The IC_{50} values were calculated from concentration-response curves by linear regression analysis.

Statistical analysis

Data are expressed as mean \pm standard error (SE). Analyses were performed in triplicate. Results are expressed as average \pm SE. Statistical comparisons were made by one-way ANOVA, followed by Tukey's test. $P < 0.05$ with GraphPad Prism $\text{\textcircled{R}}$ 4.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

MTT results showed that all the extracts decreased the viability of the cell lines in a dose-dependent manner. However, all extracts also displayed variation in biological activity that could be ascribed to the geographic region of sample collection, the phytochemical profile of the extracts, or the cell lines tested. All extracts showed a high inhibitory activity (60 to 70 %) towards leukemia cell line HL - 60 with half

maximal inhibitory concentration (IC_{50}) undetermined for Arraial do Cabo (RJ), but the IC_{50} of $300.70 \mu\text{g mL}^{-1}$ for Araranguá (SC) (Table 2). Extracts from Montes Claros (MG), Cáceres (MT) and Rio de Janeiro/UFRJ (RJ) showed a lower IC_{50} for the lymphoma cell line (Daudi) compared to that from Belém (PA), Araranguá (SC) and Arraial do Cabo (RJ) (Table 2). The extract from MG showed a still lower inhibitory activity (45 to 30 %, with IC_{50} of $493.30 \mu\text{g mL}^{-1}$) for B16F10, a metastatic murine melanoma. Even though the IC_{50} values of the extracts were higher than those observed for clinically used antineoplastic drugs, such as cisplatin (Table 2), such difference is irrelevant to this study, as *E. tirucalli* extracts are not, in any way, suggested to be a substitute for cisplatin or any other chemotherapeutic drug.

To evaluate extract composition, phytochemical analyses were performed. Based on HPLC analysis, signals from phenolic compounds represented by flavonoids and tannins were identified by chromatogram profiles of the

extracts (Figure 1 A and B). Retention times of 20.24 min (Figure 1A) and 20.39 min (Figure 1B) in chromatograms indicate flavonoids whose UV spectra presented absorbance maxima of 256 and 365 nm, and 260 and 356 nm, respectively. Retention times of 27.7 min and 32.2 min were detected for tannins for both chromatograms, showing absorbance maxima of 250 - 260 nm and 374 - 385 nm (Figure 1).

Currently, the ultra-high resolution (potentially in excess of 10^6) and accuracy ($< 1 \text{ ppm}$) of mass spectrometry, such as FT - ICR MS, allow the identification of complex organic mixtures without prior extraction or separation steps. FT - ICR MS, which is applied in such sciences as metabolomics, proteomics or petroleomics, enables analyses of complex mixtures at the molecular level. Accurate mass measurements define a unique elemental composition, e.g., ($C_xH_nN_nO_oS_s$), based on such singly charged ions as $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, $[M - H]^-$ and $[M + Cl]^-$, where M corresponds to neutral molecules [14-16].

Table 2: Antiproliferative activities of *Euphorbia tirucalli* extracts on tumor cell lines

Extract	Cell lines ($IC_{50} \mu\text{g mL}^{-1}$)		
	HL- 60	Daudi	B16F10
Cisplatin (positive control)	6.95*	14.98 *	19.43*
Montes Claros (MG)	202.49	318.27	493.30
Belém (PA)	183.38	nd	nd
Cáceres (MT)	121.01	319.70	nd
Araranguá (SC)	300.70	467.20	nd
Arraial do Cabo (RJ)	113.06	nd	nd
Rio de Janeiro/UFRJ (RJ)	202.62	393.59	nd

*Data provided by Dr A Esteves-Sousa; nd = not determined.

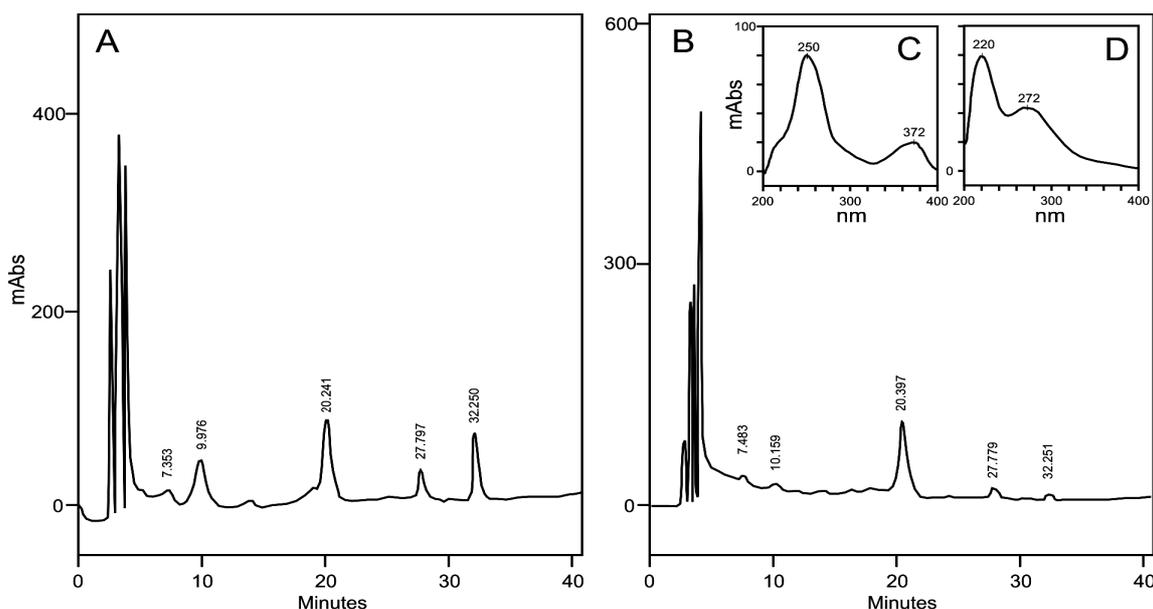


Figure 1: HPLC chromatogram profiles of *Euphorbia tirucalli* extracts at a wavelength of 240 nm (A. Montes Claros, MG; B. Arraial do Cabo, RJ). C and D. Ultraviolet spectra of tannins found in the extracts

ESI (-) FT-ICR MS spectra for ethanolic extracts of *E. tirucalli* extracts are shown in Figure 2. Here, ions are detected in deprotonated form, $[M - H]^-$, corresponding to malic acid glycosides (m/z 295.0672 and 457.1204 with $M = C_{10}H_{16}O_{10}$ and $C_{16}H_{26}O_{15}$ and DBE = 3 and 4, respectively); ellagic acid (m/z 300.9991 with $M = C_{14}H_6O_8$ and DBE = 12); **Quercetin** (m/z 447.0937 with $M = C_{21}H_{20}O_{11}$ and DBE = 5), as well as some classes of phenolic compounds like galloyltannins (m/z 483.0785 with $M = C_{20}H_{20}O_{14}$ and DBE = 11), ellagitannins (m/z 481.0628 and 633.0740 with $M = C_{20}H_{18}O_{14}$ and $C_{27}H_{22}O_{18}$ and DBE = 12 and 17, respectively) and phenylpropanoids (m/z 353.0880 with $M = C_{16}H_{18}O_9$ and DBE = 8). Acid triterpene was also detected as having lower relative intensity (m/z 455.3536 with $M = C_{30}H_{48}O_3$ and DBE = 7). Veracylglucan A, B and C (malic acid glycosides) were identified in the gel as antiproliferative and anti-inflammatory compounds in *in vitro* assays. Veracylglucan A and B detected as ions of m/z 295 and 457, respectively, were also identified in *E. tirucalli* extracts. To confirm the structures and the connectivity of the Veracylglucan A and B compounds, ESI(-) - MS/MS spectra were acquired.

Thermally assisted collision-induced dissociation (TA-CID) experiments were performed for the ion of m/z 295, which produced fragments of m/z 251 and 179 corresponding to neutral losses of CO_2 (44 Da) and CH_3COCO (72 Da, via $251 \rightarrow 179$ transition), respectively. Similar results were observed for the ion of m/z 457, producing fragments of m/z 413 (via CO_2 loss), 341 (via $413 \rightarrow 341$ transition, corresponding to CH_3COCO) and 295 (hexose loss, 162 Da). These results strongly indicate that the molecules represented by the ions m/z 295 and m/z 457 are the same as those of Veracylglucan A and B compounds found in *A. vera* gel [17].

Generally, therefore, a similar chemical profile was found among all *E. tirucalli* samples, as shown in Figure 2 and Table 3. However, quantitatively, a marked difference is indicated by the relative intensities of the $[M - H]^-$ ions of the main compounds. Some of these compounds are considered cytotoxic, suggesting the presence of a biologically active phytocomplex. However, the tumor-promoting phorbol esters, which are known to mimic 1,2-diacylglycerol, an activator of protein kinase C [18], as well as other plant toxins characteristic of Euphorbiaceae diterpenes, were not detected by the analytical

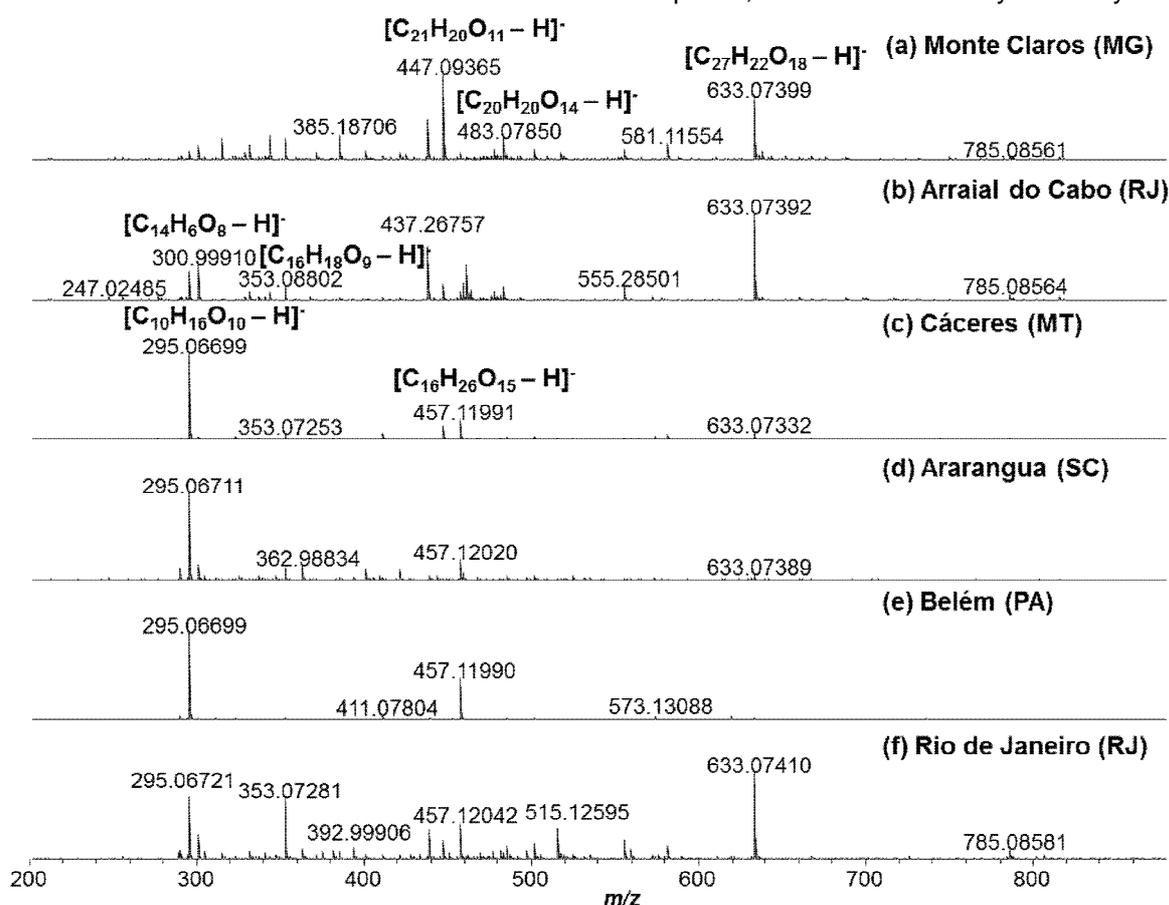


Figure 2: ESI(-)-FT-ICR mass spectra for ethanolic extracts of *Euphorbia tirucalli*

Table 3: Chemical compounds in *Euphorbia tirucalli* extracts determined from ESI(-) FT- ICR MS data

Molecular formula or M (DBE)*	MG	PA	MT	SC	Arraial do Cabo/RJ	UFRJ/RJ	Proposed structure (Class of Natural Product)
C ₁₀ H ₁₆ O ₁₀ (3)	+	+++	+++	+++	++	+++	Malic acid glycoside
C ₁₄ H ₆ O ₈ (12)	+	+	+	+	++	+	Ellagic acid (phenolics)
C ₁₆ H ₁₈ O ₉ (8)	+	+	+	+	+	+++	Mono-caffeoylquinic acid (phenylpropanoid)
C ₂₁ H ₂₀ O ₁₁ (12)	+++	nd	+	+	+	+	Quercetin (flavonoid)
C ₃₀ H ₄₈ O ₃ (7)	nd	+	nd	nd	+	nd	Acid triterpene (triterpene)
C ₁₆ H ₂₆ O ₁₅ (4)	nd	++	+	+	nd	++	Malic acid glycoside
C ₂₀ H ₁₈ O ₁₄ (12)	+	nd	nd	nd	+	+	2,3-(S)- Hexahydroxydiphenyl- D -glucose (ellagitannin)
C ₂₀ H ₂₀ O ₁₄ (11)	+	nd	nd	+	+	+	2,3- Di-O-galloyl-D-glucose (galloyltannin)
C ₂₇ H ₂₂ O ₁₈ (17)	+++	+	+	+	+++	+++	2,3-(S)- Hexahydroxydiphenyl- D -glucose (ellagitannin)

nd = not detected; +++high intensity of [M-H]⁻ ions; ++medium intensity of [M-H]⁻ ions; + low intensity of [M-H]⁻ ions

techniques employed in this study, although they can be present in minor, undetectable quantities. Table 3 summarizes the presence of chemical compounds detected in *E. tirucalli* extracts and their relative quantitative variations.

DISCUSSION

Based on the results, it can be concluded that differences in antiproliferative activity of *E. tirucalli* extracts against a given cell line likely result from variations in the production of secondary metabolites, which can, in turn, be influenced by the variations in geographic/climatic conditions found throughout Brazil. Brazil is a tropical country divided into about six different climate zones. More specifically, PA is located in northern Brazil and presents high temperatures and precipitation throughout the year. In contrast, MG and MT are located in southeastern and Central-Western Brazil, respectively, and while these regions also experience high temperature and humidity, most rainfall is concentrated in the summer. Still different, SC is located in southern Brazil which has a temperate climate with low temperatures in the winter. Finally, RJ, located in southeast Brazil, is a city on the Atlantic coast that is influenced by the humidity of the Atlantic air mass. These data suggest considerable climatic variation and, correspondingly, very different

growing conditions.

The analysis of ultraviolet profiles revealed the predominant presence of hydrolysable tannins, specifically, gallotannins and ellagitannins, whose spectra presented absorbance maxima of 220 and 272 nm, respectively [13], and tannins in the UV spectrum between 250 and 372 nm at a retention time of 32.2, suggesting the presence of ellagic tannins belonging to the gallagyl group [12] or ellagic acid derivatives. The flavonoid glycosides quercetin and rutin are known to be present in *E. tirucalli* aerial parts [11].

The presence of some compounds is associated with the treatment of cancer, such as triterpene euphol from *E. tirucalli*, which regulates the cell cycle of cancer cells [20], and derivatives of phloroglucinol, Ebracteolatin A and B from *E. ebracteolata*, which induced apoptosis in human hepatoma cell line [21]. The malic acid glycosides, as detected in this study through ESI (-) FT-ICR mass spectra of ethanolic extracts of *E. tirucalli*, have also been found in *Aloe vera* gel [17], a plant popularly known as *babosa* and indicated for treatment of cancer and other ailments.

Based on the use of aveloz as a popular anticancer phytomedicine, the present study aimed to compare the antiproliferative activity of *E. tirucalli*, a plant which grows in a wide variety

of geographic and climatic conditions. We asked if such conditions would influence the production of secondary metabolites, thus changing the chemical composition of samples and, hence, affecting its medicinal properties in the context of uniform dosage across all Brazilian states. Tannins have already been isolated from *E. jolkini*, *E. prostrata* and *E. hirta*, as well as from *E. tirucalli* plants [13]. The antitumor activity of tannins and hydrolysable tannins (1-O-galloyl castalagin, casuarinin and 2-O-galloylpunicalin) against different cancer cell lines has already been described [13,22]. Interestingly, plants collected in southeastern Brazil showed higher production of ellagitannins (peak 633.07399). The antiproliferative activity of malic acid glycosides, like Veracylgucan A and B, was described for *A. vera* gel [17]. Except for one sample from Minas Gerais State, all studied samples showed considerable production of Veracylgucan A (peak 295.06721). The lower response to the lymphoma cell line (Daudi) by extracts from Montes Claros (MG), Cáceres (MT) and Rio de Janeiro/UFRJ suggests that melanomas are chemo- and radioresistant tumors [19].

CONCLUSION

The findings of this study show that extracts of *E. tirucalli* are cytotoxic against different cancer cell lines, thus generally supporting its popular use as an anticancer folk medicine. The data also show differences in antiproliferative activity among extracts obtained from different regions of Brazil, which likely results from the different concentrations of compounds in the extracts. The predominant compounds in the extracts are malic acid glycosides, including veracylgucan and ellagitannin. To fully understand the mechanism(s) of action involved in the antiproliferative activity of *E. tirucalli*, further investigations are required.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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