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SEASONAL EFFECT ON *Euphorbia tirucalli* L. CYTOTOXICITY

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ABSTRACT:

Many studies have reported on the use of *Euphorbia* species against cancer. Tinctures of *Euphorbia tirucalli* L., popularly known as aveloz, have been widely used in Brazilian traditional medicine to treat cancer. However, the influence of seasonal variations on the cytotoxic activity of *E. tirucalli* ethanolic extracts (tinctures) has not been investigated. To accomplish this in the present work, aerial parts of *E. tirucalli* were collected each month in an experimental garden in Rio de Janeiro City. Effects of seasonality on the in vitro cytotoxic activity of E. tirucalli were evaluated. Tinctures were prepared by maceration in ethanol 70%, and qualitative phytochemical analyses were carried out using high-performance liquid chromatography (HPLC/UV/DAD). Cytotoxicity was evaluated in vitro against human lymphoma cells (Daudi) by MTT assay. The presence of triterpenes, hydrolysable tannins and flavonoids was detected in the tinctures. Tinctures prepared from plants collected seasonally showed concentration-dependent cytotoxicity against the analyzed cell line. Therefore, it can be concluded that environmental conditions based on seasonal changes did induce variability in phenolic compounds content and, hence, cytotoxic activity of aveloz on tumor cells.

Keywords: Anticancer, Traditional medicines, Phenolics compounds, Seasonal variation effect.

RESUMO:

Muitos estudos têm abordado o uso de espécies de *Euphorbia* contra o câncer. Tinturas de Euphorbia tirucalli L., conhecida popularmente como aveloz, são amplamente utilizadas na medicina tradicional no Brasil para tratar o câncer. No entanto, a influência da sazonalidade na atividade citotóxica de extratos etanólicos (tinturas) de *E. tirucalli* ainda não foi investigado. Em vista disso, no presente trabalho, partes aéreas de *E. tirucalli* foram coletadas mensalmente em um jardim experimental na cidade do Rio de Janeiro. Foram avaliados os efeitos da sazonalidade sobre a atividade citotóxica in vitro de *E. tirucalli*. As tinturas foram preparadas por maceração em etanol 70% e as análises fitoquímicas foram feitas utilizando Cromatografia Liquida de Alta Eficiência (CLAE/UV/DAD). A citotoxicidade foi avaliada pelo ensaio in vitro do Methyl Thiazolyl Blue (MTT) contra células de linfoma humano (Daudi). Foram detectados nas tinturas triterpenos, taninos hidrolisados e flavonoides. As tinturas preparadas a partir de plantas coletadas ao longo do ano mostraram citotoxicidade dependente da concentração. Portanto, pode-se concluir que as condições ambientais, como as mudanças sazonais, induziram variabilidade no conteúdo de substâncias fenólicas e, portanto, na atividade citotóxica de aveloz contra células tumorais.

Palavras-chave: anticancer, medicina tradicional, substâncias fenólicas, variação sazonal

INTRODUCTION

According to the World Health Organization, cancer causes 7 million deaths every year, totaling 12.5% of deaths worldwide. Chemotherapy is the most common cancer treatment. However, clinical data reveal that cancer cells are resistant to a variety of structurally unrelated chemotherapeutic drugs that do not share the same target cell, resulting in multidrug resistance (MDR) (Gottesman & Ling, 2006). Therefore, continuous studies on the medicinal properties of plant extracts have been extensively employed aiming to elucidate and confirm chemotherapeutic alternatives (Solowey et al., 2014).

Euphorbia tirucalli L. (*Euphorbiaceae*), commonly known as aveloz, is a succulent and lactescent shrub belonging to xerophytic vegetation of wide occurrence in Brazil, and it has been popularly used in tropical countries for its antitumor properties. The traditional and popular use of aveloz is commonly through diluted latex or tinctures which are produced from fresh plant material in contact with alcoholic solution (Silva et al., 2007, Upadhyay et al., 2010, Santana et al.,

2014). Scientific studies have confirmed the effective activity of *E. tirucalli* and E. hirta against cancer, e.g., the inhibition of ascitic tumors in mice (Betancur-Galvis et al., 2002, Valadares et al., 2006, Sharma et al., 2014, Santos et al., 2016). Some authors showed that *E. tirucalli* latex inhibits proliferation and gene expression of larynx cancer (Franco-Salla et al., 2016). Other authors have found that *E. tirucalli* exhibits cytotoxic properties in a dose- and time-dependent manner in different cell lines (Munro et al., 2015, Santos et al., 2016, Caxito et al., in press). This herb presents modulatory effect, as observed in different assays, and this effect has also been verified in its isolated lectin known as Eutirucallin (Santana et al., 2014, Franco-Salla et al., 2016). Phytochemical constituents with pharmacological properties isolated from the *Euphorbia* genus are generically found in leaves and latex of almost all species. For *E. tirucalli*, Liquid Chromatography-Mass Spectroscopy (LC-MS) verified such secondary metabolites as terpenes and flavonoids (Choene & Motadi, 2016).

Brazil is a tropical country with different climatic conditions created by variations in temperature and pluviosity. Specifically, the climate in Rio de Janeiro City is typically marine tropical owing to the long coastline and preponderance of sunlight and rain along the year. In general, most Brazilian states share a two-season pattern: wet and dry.

These seasonal variations interfere with the production of a plant's secondary metabolites that afford it with bioactive characteristics (Ramakrishna & Ravishankar, 2011, Sampaio et al., 2016). In order to understand the effects of seasonal variations on the biological activity of E. tirucalli, tinctures were obtained from the raw material at different times of the year, and in vitro cytotoxicity against Daudi cells, a Burkitt's lymphoma cell line, was determined by MTT assay.

MATERIAL AND METHODS

Areas of collection and plant materials

E. tirucalli samples were collected from March 2006 to February 2007 in Rio de Janeiro City (Rio de Janeiro, Brazil). Figure 1 shows the climatic features, temperature,pluviosity, and number of rainy days for each collection month based on data obtained from the National Institute for Meteorology (INMET, Brazil). The wet season runs from January to March and September, and the dry season runs from June to August and October to December. Average annual rainfall is around 9.25 mm, and average annual temperatures range between 20.9 °C (min) and 25.7 °C (max). *E. tirucalli* samples were identified, and they were deposited under accession number RFA-31675 in the Herbarium of the Biology Institute, Federal University of Rio de Janeiro (Universidade Federal do Rio de Janeiro, UFRJ).

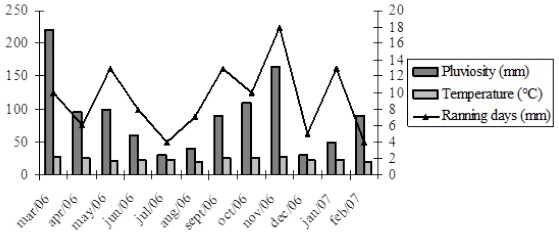

Figure 1. Summary of climatic conditions where *Euphorbia tirucalli* L. was collected during the study period in Rio de Janeiro City. Data were obtained from National Institute for Meteorology (INMET, Brazil).

Preparation of ethanolic extracts (tinctures)

E. tirucalli aerial parts were collected in the morning, then, the fresh plant material was weighed and submitted to tincture preparation as follows. Twenty grams of plant material were immersed in 100 mL of 70% ethanol and allowed to macerate in a shaker for a week. Crude extracts were filtered and dried through evaporation at 60ºC in a rotatory

evaporator, followed by freeze-drying.

Chtomatography Analysis
Analysis by High-performance Liquid Chromatography (HPLC)

HPLC-UV-DAD analyses were performed in a Shimadzu apparatus equipped with a SPD-M10A 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 µ L of this solution were injected. 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: MilliQ water (A) and methanol (B) with gradient of 50% to 90% B, in 40 minutes, at a flow rate of 1 mL/min. A photodiode detector focused at 240 nm was used. Component relative percentages were calculated from the peak area current by an automated integrator. Flavonoids and tannins were detected on the basis of their retention times and UV-spectra compared to the authentic standard through retention times and previous studies reporting on the phytochemistry of *E. tirucalli* (Yoshida et al., 1991). All chemicals used in the analysis, such as methanol, were of HPLC grade and were purchased from Merck. MilliQ water was utilized for HPLC mobile phase and sample preparation. Analyses were performed in triplicate.

Analysis by Thin Layer Chromatography (TLC)

After its isolation from avelóz, the flavonoid quercitrin was used as a standard for qualitative analyses using TLC. To identify flavonoids, 40 µl of each solution of avelóz tincture were spotted on TLC plates (silica gel 60 F254 nm, Merck) and developed with ethyl acetate: ethanol: acetic acid:H2O (16:1.5:1:1). Spots were detected at 254 and 366 nm wavelengths and revealed by spraying the sheets with NP/PEG (1% methanolic diphenylboric acid/β-Ethanolamine ester/polyethylene glycol) reagent (Fluka Chemie, Switzerland). TLC was observed under UV spectrum at 254 and 360 nm.

The presence of tirucallol triterpene, commonly found in *E. tirucalli* extracts, was isolated from avelóz latex and used as a standard in TLC analysis. To identify triterpenes, 40µl of each solution of avelóz tincture were spotted on TLC plates (silica gel 60 F254 nm, Merck) and developed with hexane:ethyl acetate (8:2). TLC plates were observed under UV lamps at 254 and 360 nm spectra. After this process, spots were evidenced with sulfuric acid 5% and anisaldehyde 5% in absolute ethanol under heating. For use, the material was dissolved in DMSO (Sigma, St. Louis, MO) and then diluted in culture medium.

Human cell lines

Daudi cells (Burkitt's lymphoma cell line) were used. RPMI was used to cultivate the cancer cells. Medium was supplemented with 10 % FBS, 100 µg/ml of penicillin, 100 µg/ml of streptomycin, 2 mM glutamine and 0.07% NaHCO3. The cultures were maintained at 37°C in humidified 5% CO2 atmosphere.

Cell viability assay

Cell viability was assessed by MTT (Methyl-Thiazol Tetrazolium), as described by Mosmann (1983). After 24 h resting, plated cells (2 x 104/well) were treated with medium, the desired concentrations of extracts (62.5, 125, 250, 500 µg/mL), or dimethyl sulfoxide (DMSO) in the same concentrations, and incubated for another 48 h. After incubation, each well received 2.5 mg/mL of MTT (3-4, 5-dimethylthiazo-2yl-2,5-diphenyltetrazolium

bromide), and the plates were incubated for an additional 4 h at 37°C. The medium was removed, and the crystals of reduced formazan were dissolved with 150 µL of DMSO. Absorbance was determined at 570 nm with a microplate reader (BenchMark, Bio-Rad, Hercules, CA). Effects of the tinctures on cell viability were calculated, using cells treated with DMSO as control.

Statistical analysis

For the experiments of cell proliferation, data were expressed as a percentage. Analyses were performed in triplicate. Results are expressed as average ± SE. Statistical comparisons were made by one-way ANOVA, followed by the Tukey's test. $P < 0.05$ with Graph Pad Prism 4.0. (Graph Pad Software, Inc.)

RESULTS

Analysis of cytotoxic activity

The cytotoxic activity of tinctures on Daudi cells occurred in a dose-dependent manner (Figure 2). The main effective concentration (IC50) of *E. tirucalli* tinctures was calculated through the curve of linear trend. Monthly collections of avelóz from samples taken from an experimental Rio de Janeiro garden showed differences in the cytotoxic potential of *E. tirucalli* along the year (Figure 2). Specifically, tinctures from plants collected in April 2006 and January 2007 presented reduced inhibition compared with other months at 47.6% and 46.3%, respectively. The cytotoxic activity in other months averaged approximately 60%, and in June, the percent of inhibition was 71.8%, representing the best inhibition against the Daudi cell line. Significant differences in cytotoxic activity were observed when comparing the wet months of April 2006 and January 2007 with the relatively dry month of June 2006.

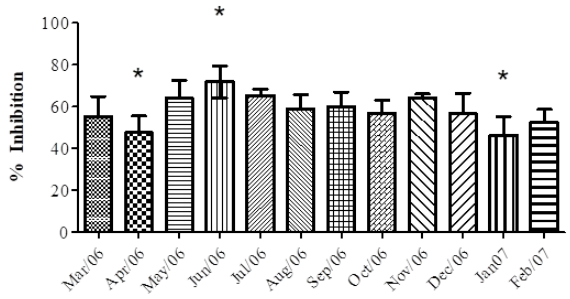

Figure 2. Effects of *Euphorbia tirucalli* L. ethanolic extracts (500 µg/mL) collected monthly in Rio de Janeiro City on tumor cell viability of Daudi cell line. Results are expressed as % control ± SE of at least three experiments performed. *Indicates statistical differences at $p < 0.01$.

Detection of phenolic compounds by TLC and HPLC

Tinctures were submitted to thin layer chromatography to investigate the presence of flavonoids and tannins. Results demonstrated that all samples presented the flavonoid quercitrin, Rf = 0.71, and the triterpene tirucallol, Rf = 0.76.

UV spectra obtained from HPLC-UV showed the presence of hydrolysable tannins and flavonoids in all extracts analyzed for each month (Figure 3). Based on our chromatographic data, we observed a larger variety of phenolic compounds in extracts from plants collected in June 2006. Figure 3 shows the replicate signals obtained by HPLC from each tincture from plant material collected in each month of the study. These compounds were detected as having between 7.87 min and 22.17 min retention time (RT), among them, ellagic tannins and flavonoids were detected by UV absorbance. A high content of flavonoids was observed in samples collected in all months. The relationship between relative areas (%) of flavonoids/tannins compounds was 3.9% for

samples collected in June.

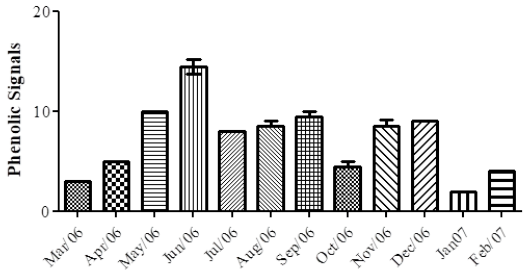

Figure 3. Relative content of phenolic compounds (%) detected in Euphoria tirucalli L. tinctures by their chromatographic signal, as determined by HPLC analysis on the basis of retention time and UV-spectra (between 7.87 min and 22.17 min) compared to the authentic standard after replicate extractions and analysis.

DISCUSSION

In Brazil, we do not find significant climatic variations among the months, essentially because the seasons are not as well defined as those in temperate zones. However, changes in temperature and pluviosity allow us to distinguish between summer and winter seasons. Specifically, during the course of the study in Rio de Janeiro City, high variability in rainy periods is noted along the year, with most rain occurring from October to April,. Comparing climatic parameters, such as temperature and rainfall, the most significant climatic seasonal variation in Rio de Janeiro City was defined by rainfall. These climatic differences suggested that cytotoxic activity of tinctures of *E. tirucalli* might be altered along wet and dry seasons. Indeed, phenolic compounds, flavonoids and tannins all presented differences in chromatographic signals, depending on the month of collection. Several studies with different Euphorbia species have detected and/or isolated flavonoids and tannins (Jassbi, 2006, Caxito et al., in press). In the present study, high content of these phenolic compounds was observed in plant material collected in June, which coincides with greater inhibition of Daudi proliferation by tinctures. Studies have shown that different concentrations of phytochemical compounds from *E. tirucalli* extracts were responsible for producing antiproliferative effect on cancer cells (Choene & Motadi., 2016). However, quantitative and qualitative variations in phenolic metabolites along the year have also been observed in several plant species (Scognamiglio et al., 2015). Tannins have been studied, and different biological effects, such as chemopreventive action against prostate cancer and inhibition of tumor-associated angiogenesis, were noted. In the *Euphorbiaceae*, tannins have already been isolated from *E. jolkini*, *E. prostrata* and *E. hirta*, as well as from *E. tirucalli* (Yoshida et al., 1991). Flavonoids are a group of natural products present in a wide variety of plants and exhibit a broad range of antiviral, anti-inflammatory, antioxidant and antitumor activities (Blank et al., 2004). Huber et al. (2009) found that seasonal variations of flavonoids in Brazilian vegetable sources tend to be higher in the summer. In another study, the antioxidant activity of *Pistacia atlantica* leaves and its phenolic compounds, including flavonoids and condensed tannins, were all affected by the harvest month (Ben Ahmed et al., 2017).The medicinal activities of flavonoids from Euphorbiaceae are well documented (Dinkova-Kostova et al., 1998).

In the present study, we used TLC analysis, and the triterpene tirucallol was detected in all tinctures of *E. tirucalli*. Some studies have shown that this triterpene isolated from the leaves of *E. pulcherrima*, *E. lactea* and *E. tirucalli* presents cytotoxic activity against Ehrlich Ascites tumor (Fernandez-Arche et al., 2010).

Indeed, as verified in plants collected in June 2006, the dry season, cytotoxic activity was higher as shown in Figure 4 compared to tinctures from plants collected in other months.

As noted, this result coincides with the initiation of the dry season in Rio de Janeiro. This species can survive in a wide range of habitats, but it originated from tropical East Africa, and it is a succulent, cactus-like plant, showing the adaptations needed to grow in arid and mesophytic zones (Upadhyay et al., 2010). Therefore, based on the collective results of the present study, seasonality does play a role in altering the potency of secondary metabolites produced by this species. Other studies have corroborated these findings. For example, in studies with leaf extracts of *Bauhinia forficata*, higher values of antioxidant activities were observed in plants collected during dry periods in Brazil (Ruela, 2007). Changes in cytotoxic activity have also been verified from extracts of *Alstonia scholaris* in HeLa cancer cells, depending on the season of collection and extraction (Jagetia et al., 2005). Environmental differences and seasonal conditions modulated secondary metabolite content of total phenolic compounds and flavonoids in four juniper species (Tavares et al., 2013).Therefore, Big Pharma should take into consideration the likely fluctuation in pharmacological properties in secondary metabolites relative to seasonality as it searches for new sources of bioactive compounds (Ramakrishna & Ravishankar, 2011, Tavares et al., 2013). In general, while the variation in cytotoxic activity was slight for tinctures of *E. tirucalli* according to temperature along the year, differences based on pluviosity were significant (Figure 4).

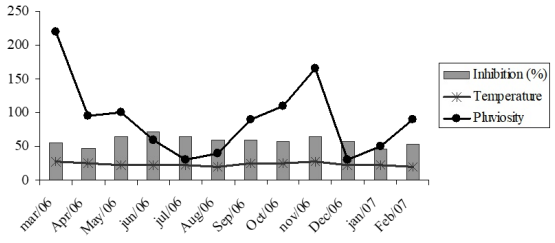

Figure 4. Overlap of temperature and pluviosity and cytotoxic activity of Euphorbia tirucalli tinctures on tumor cell viability of Daudi cell line.

CONCLUSION

Through biological assays, it was possible to observe the cytotoxic activity of the *E. tirucalli* tinctures. E. tirucalli tinctures from plants collected in June, a dry period of year, presented higher cytotoxic activity. However, it was not possible to observe the higher effects of tinctures in other dry periods. Chromatographic signals of such chemical constituents as tannins and flavonoids varied seasonally. In June, samples presented the higher content of phenolic compounds, while in January and April (wet season), they showed the low phenolic content. Such variations in secondary metabolites indicate that users of the popular medicinal plant aveloz should be cautious since potency of biological activity depends on chemical composition which, in turn, depends, to some extent, on the season of collection and extraction of the tinctures.

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