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In vitro cytotoxicity of *Duguetia* sp extracts on Ehrlich tumor cells

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Abstract: The genus *Duguetia*, of the Annonaceae family, includes around 100 species that have been used in folk medicine to treat several diseases. Its phytochemical compounds have been researched for their antifungal, antioxidant, antigenotoxic, anti-inflammatory and cytotoxic properties. Therefore, the objective of the present study was to conduct a preliminary study on the biological effects of *Duguetia* sp extracts by evaluating their in vitro cytotoxic effect on different cell types. To prepare the methanolic extract (ME), the leaves were macerated with 80% methanol. Part of the ME was dissolved in 10% phosphoric acid, and the acidic aqueous solution was partitioned with dichloromethane. The organic phase was evaporated to obtain an acetogenin-rich extract (ACE). The extracts were diluted in RPMI culture medium, supplemented with 20% fetal bovine serum, and added to Erhlich tumor cells or mice spleen cells at different concentrations: the ACE extract at 1.35 mg mL^{-1}, 0.67 mg mL^{-1} or 0,34 mg mL^{-1}, and the ME extract at 1.40 mg mL^{-1}, 0.70 mg mL^{-1} or 0,35 mg mL^{-1}. After 24 h, cytotoxicity analysis was performed using the Trypan Blue exclusion method. The results demonstrated that the extracts are cytotoxic at all concentrations to Ehrlich tumor cells and mice spleen cells. According to the results, we concluded that the extracts of *Duguetia* sp had a cytotoxic effect on the studied cells in vitro. This was the first study to report the biological effect of this plant genus in this type of cells; however, further study is needed to determine the current species used and the compounds present in the extracts.
Keywords: *Duguetia* sp, phytotherapic, Ehrlich tumor, cytotoxicity

Introduction

The use of medicinal plants in simple or complex medicinal formulations is an integral part of popular culture worldwide and has been accompanied by the development of civilizations (Akram et al., 2014). Several studies have reported the biological effects of compounds extracted from plants, fungi, marine animals, amphibians, and microorganisms (Kaneno et al., 2004; Martins et al., 2008; Ferreira et al., 2013; Sultana et al., 2014; Albiero et al., 2016).

Several pharmaceutical products that are currently used, such as reserpine, deserpidine, vinblastine, and paclitaxel are medicinal plant-based drugs (Sultana et al., 2014). The National Cancer Institute in the United States has evaluated approximately 114,000 natural extracts with anticancer activity (Sultana et al., 2014). In Brazil, unconventional therapeutic agents are regulated by law, which legitimizes their use in therapies such as acupuncture, phytotherapy, and homeopathy (Brasil, 2013).

The scientific community recommends that products of unknown activity be initially evaluated for their biocompatibility through *in vitro* cell culture studies (Martins et al., 2009). *In vitro* cytotoxicity tests involve the culture of mammalian cells that are either in direct or indirect contact with a compound or material, and monitoring for cellular alterations (Rogero et al., 2003).

One of the most commonly used parameters to evaluate cellular changes is cell viability, which can be visualized using vital dyes such as neutral red or Trypan blue (Rogero et al., 2003; Oliveira et al., 2010). The main objective of *in vitro* cytotoxicity assays is to facilitate the study of cell behavior in a controlled environment free of the complexity of the living organism and in an easy, fast, and inexpensive way (Martins et al., 2009). Following *in vitro* studies, the material or compound may enter animal studies and human clinical trials (Rogero et al., 2003; Martins et al., 2009).

The genus *Duguetia* from the family Annonaceae contains approximately 100 plant species that have been used in folk medicine for treating several diseases (Pérez & Cassels, 2010; Rodrigues et al., 2015). This genus comprises mostly small, understory trees growing almost exclusively in the tropics of South America, with a

small extension across the Panama Isthmus (Pérez & Cassels, 2010).

Many studies have been conducted on the secondary metabolites present in different parts of *Duguetia* plants, such as essential oils, aromatic compounds, monoterpenes, diterpenes, triterpenes, flavonoids, alkaloids and acetogenins (Pérez & Cassels, 2010; Sousa et al., 2012; Rodrigues et al., 2015). Secondary metabolites are substances with a generally complex structure playing several roles in the adaptation of plants to the environment (Pinho et al., 2016). Nowadays, these substances have been attracting interest for their pharmacological potential, such as their antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, and antimicrobial properties (Pinho et al., 2016).

Therefore, the objective of this study was to evaluate the *in vitro* cytotoxic effects of *Duguetia* sp extracts in culture with Ehrlich tumor cells and mice spleen cells. This is the first study on the effects of *Duguetia* sp extract on these types of cells. The findings of this study are important for corroborate the reported biological action of *Duguetia* sp and its ethnobotanical use.

Methods

Ethical Principles

This study was submitted to the Ethics Committee on Animal Use (CEUA Protocol nº 23108.702149/13-0) of the Federal University of Mato Grosso (UFMT) and was approved, as it follows the required ethical and legal principles.

Animals

Male Swiss mice (n=2), aged 40 to 50 days, were obtained from the Central Animal Facility of the Federal University of Mato Grosso – UFMT, Cuiabá, Brazil. The animals were kept in polypropylene boxes with xylan substrate (Suprimart Mercantil, Itaquaquecetuba, SP, Brazil) at 22°C with 12/12h light/dark cycles. The mice received filtered water and pelleted feed (Purina, St. Louis, Missouri, USA) *ad libitum*.

Duguetia sp extracts

Leaves of *Duguetia* sp, Annonaceae, were collected in Sorriso (GPS coordinates 11°53´ 53.5" S and 55°39´45.7" W), in the Middle-North of Mato Grosso State, which is the transition region of the Amazon and Cerrado biomes. Botanical identification was performed by Dr. Aline Fernandes Pontes Pires of the Federal University of Mato Grosso (Universidade Federal do Mato Grosso - UFMT) at Sinop, Mato Grosso State (Brazil). Plant collection was authorized by SISBIO (Brazilian Government's Authorization and Information in Biodiversity System, process A9AD5FF). Dried and powdered leaves of *Duguetia* (293 g) were macerated with 80% methanol (1:10 m / v), over a period of 7 days. After this period, the solvent was eliminated using a rotative evaporator, leaving the methanolic extract (ME). Part of the ME was dissolved with 10% phosphoric acid, and then the acidic aqueous solution was partitioned five times with dichloromethane. The organic phase was dried with anhydrous sodium sulphate and concentrated to give an acetogenin-rich extract (ACE). The extracts were evaluated by thin layer chromatography (TLC) using aluminum-backed (Merck, silica gel 60 F254). The plates were sprayed with Kedde's Reagent which provides a positive test to identify the α,β-unsaturated-γ-lactone moiety commonly found in annonaceous acetogenins. For alkaloids, an orange and brown coloration was observed after the sprinkling of Dragendorff's reagent (Wagner;Bladt, 1995).

For *in vitro* analysis, the extracts were diluted in complete medium (RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, Cultilab, Campinas, SP, Brazil) at 1.35 mg mL^{-1}, 0.67 mg mL^{-1} or 0,34 mg mL^{-1} for the ACE extract and at 1.40 mg mL^{-1}, 0.70 mg mL^{-1} or 0,35 mg mL^{-1} for the ME extract.

Total spleen cell suspension

The spleen cell suspension was obtained by teasing the spleens on a sterile fine nylon screen in RPMI 1640 medium (Cultilab, Campinas - SP, Brazil), according to the protocol described in Castoldi et al. (2006). The cell suspension was centrifuged at 1,500 rpm for 10 min and suspended in 1 mL of complete medium. The concentration of splenic mouse cells was adjusted to $4x10^6$ viable cells mL^{-1} using the Trypan blue exclusion test (Albiero et al., 2016), and a minimum viability of 70% was used as a cutoff.

Ehrlich tumor cells

Ehrlich tumor cells were kindly provided by Rondon Tosta Ramalho, Ph.D., from the Federal University of Mato Grosso do Sul (UFMS), Campo Grande, Brazil. The cells were maintained by intraperitoneal inoculation (ascitic form) in Swiss mice, every 7 days for a period of 2 weeks. The ascitic fluid of animals with the ascitic form of the Ehrlich tumor was aspirated from the peritoneum, and tumor cell suspensions were prepared in sterile phosphate-buffered saline to obtain a final concentration of $4x10^6$ viable cells mL^{-1}. Viability, assessed by the Trypan Blue dye exclusion method, was always found to be at least 70%.

Cytotoxicity assay by Trypan blue exclusion method

The toxic effect of *Duguetia* sp extracts were determined by measuring the viability of both spleen cells and Ehrlich tumor cells (Strober, 2015). Two animals were used (male Swiss mice) to prepare the spleen cell suspension, according to the protocol described previously. One animal bearing the ascitic form of the Ehrlich tumor was the source of the tumor cells. The cells (at a concentration of $4x10^6$ cells mL^{-1}) were distributed in triplicate (50 µL/well) into a 96-well flat-bottomed microculture plate, and the effect of the extracts was tested using three

serial dilutions (50 µL/well), which started at 1.35 mg mL^{-1}, for the ACE extract and 1.4 mg mL^{-1}, for the ME extract. The cells in the basal control group were distributed into 50 µL/well of complete medium without extracts. The plates were cultured for 24 h at 37 °C under 5 % CO_2. Then, cell viability was assessed by the Trypan blue exclusion method in a Neubauer chamber. The percentage of cell viability (% CV) was calculated using the following equation: % CV = [(viable cell nº x 100)/(viable cell nº + die cell nº)].

Statistical analysis

The statistical analysis was performed using the Graphpad InStat software (San Diego, California-USA). Analysis of variance (ANOVA) and Tukey-Kramer tests were employed. Differences were considered significant when the probability of error was less than 5 % ($p \leq 0.05$).

Results and Discussion

Figures 1 (ACE extract) and 2 (ME extract) show the effects of *Duguetia* sp extracts on cultures of Ehrlich tumor cells and mice spleen cells.

The ACE extract reduced the % CV of Ehrlich tumor cells (Figure 1A, *Control:* 90.29 ± 2.88; *1.35 mg* mL^{-1}: 75.93 ± 6.12; *0.67 mg* mL^{-1}: 77.53 ± 5.01; *0.34 mg* mL^{-1}: 78.94 ± 2.59, $p \leq 0.05$) and spleen cells (Figure 1B, *Control:* 70.69 ± 8.63; *1.35 mg* mL^{-1}: 46.17 ± 9.02; *0.67 mg* mL^{-1}: 35.76 ± 20.08; *0.34 mg* mL^{-1}: 19.97 ± 4.39, $p \leq 0.05$) at all dilutions tested.

The ME extract reduced the % CV of Ehrlich tumor cells only at the highest concentration (Figure 2 A, *Control:* 87.08 ± 6.57; *1.40 mg* mL^{-1}: 58.72 ± 6.73, $p \leq 0.05$). For spleen cells, the results are not presented because the control group showed viability at less than 70%. The figures also show the absolute number of viable (Figure 1 C; Figure 1 D, and Figure 2 B) and dead cells (Figure 1 E, Figure 1 F, and Figure 2 C).

The % CV reduction due to the ACE extract was followed by an increase in the number of dead Ehrlich tumor cells (Figure 1 E. *Control:* 10.33 ± 2.58; *1.35 mg* mL^{-1}: 24.50 ± 9.33; *0.67 mg* mL^{-1}: 23.17 ± 8.64; *0.34 mg* mL^{-1}: 22.67 ± 3.45, $p \leq 0.05$). For mice spleen cells, a reduction in viable cells (Figure 1D. *Control:* 33.00 ± 12.64; *0.67 mg* mL^{-1}: 11.17 ± 4.31; *0.34 mg* mL^{-1}: 9.17 ± 3.31, $p \leq 0.05$) and an increase in dead cells (Figure 1F. *Control:* 14.67 ± 8.09; *0.34 mg* mL^{-1}: 36.67 ± 10.11, $p \leq 0.05$) were observed after ACE extract treatment with both the 0.67 mg mL^{-1} and 0.34 mg mL^{-1} doses.

Treatment with ME extract showed reduction of % CV at 1.4 mg mL^{-1}. This effect was followed by a reduction in the number of viable (Figure 2 B. *Control:* 90.00 ± 8.97; *1.40 mg* mL^{-1}: 49.83 ± 9.45, $p \leq 0.05$) and an increase in dead (Figure 2 C. *Control:* 13.50 ± 7.48; *1.40 mg* mL^{-1}: 36.00 ± 12.33, $p \leq 0.05$) Ehrlich tumor cells.

The results of this study demonstrate that ACE extract is toxic to Ehrlich tumor cells at all concentrations, and that ME extract is only toxic to Ehrlich tumor cells at the highest concentrations. The cytotoxicity effect was observed in the form of a reduction of % CV and an increase in the number of dead cells.

Although we have not yet identified the species from which we obtained our extract, this is the first study on the effects of *Duguetia* sp extract on these types of cells. It corroborates the reported biological action of *Duguetia* plants and their ethnobotanical use.

The cytotoxic effects of *Duguetia* plants have been evaluated in different models (Coelho et al., 2011; Sousa et al., 2012; Silva et al., 2012; Silva et al., 2013; Rodrigues et al., 2015; Pinho et al., 2014). Silva et al. (2012) have demonstrated via the lysogenic induction test (SOS-Inductest) and mouse bone marrow micronucleous assay that *Duguetia furfuracea* lyophilized leaf extract has cytotoxic activity. The hydroalcoholic extract of *D. furfuracea* leaves has been shown to cause toxicity in *Drosophila melanogaster* after 7 days of exposure, with a reduction of viability that was associated with increasing of oxidative stress and a reduction of antioxidant enzymes in flies (Pinho et al., 2014).

The essential oils from barks of *Duguetia lanceolata* were shown to be toxic against the brine shrimp *Artemia salina* and demonstrated antimicrobial activity against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Candida albicans* (Sousa et al., 2012). In addition, essential oil produced from leaves of *Duguetia gardneriana* shows cytotoxic and antitumoral effects against HepG2 and B16-F10 tumor cells (Rodrigues et al., 2015).

Taken together, these studies corroborate the results shown in Figure 1 and Figure 2.

Growing Ehrlich tumor cells with ACE extract (Figure 1) showed a reduction of % CV and an increase of the absolute number of dead cells, at all concentrations. However, the absolute number of viable cells and the number of total cells (data no shown) was not altered. Based on this, it can be concluded that ACE extract exhibits cytotoxic effects against Ehrlich tumor cells with induction of cellular death and damage to cell membranes. ME extract also led to a reduction of % CV and an increase in dead cells, but only at the 1.4 mg mL^{-1} concentration (Figure 2).

Loss of membrane integrity is a late event relative to the destruction of cellular organelles and chromatin (Elia et al., 1993). Trypan Blue dye is excluded by living cells with intact cell membranes, but can be absorbed by dead cells with damaged membranes, which turn blue after dye application (Adan et al., 2016). However, necrosis or apoptosis cannot be distinguished using the Trypan Blue dye exclusion method (Strober, 2015; Adan et al., 2016).

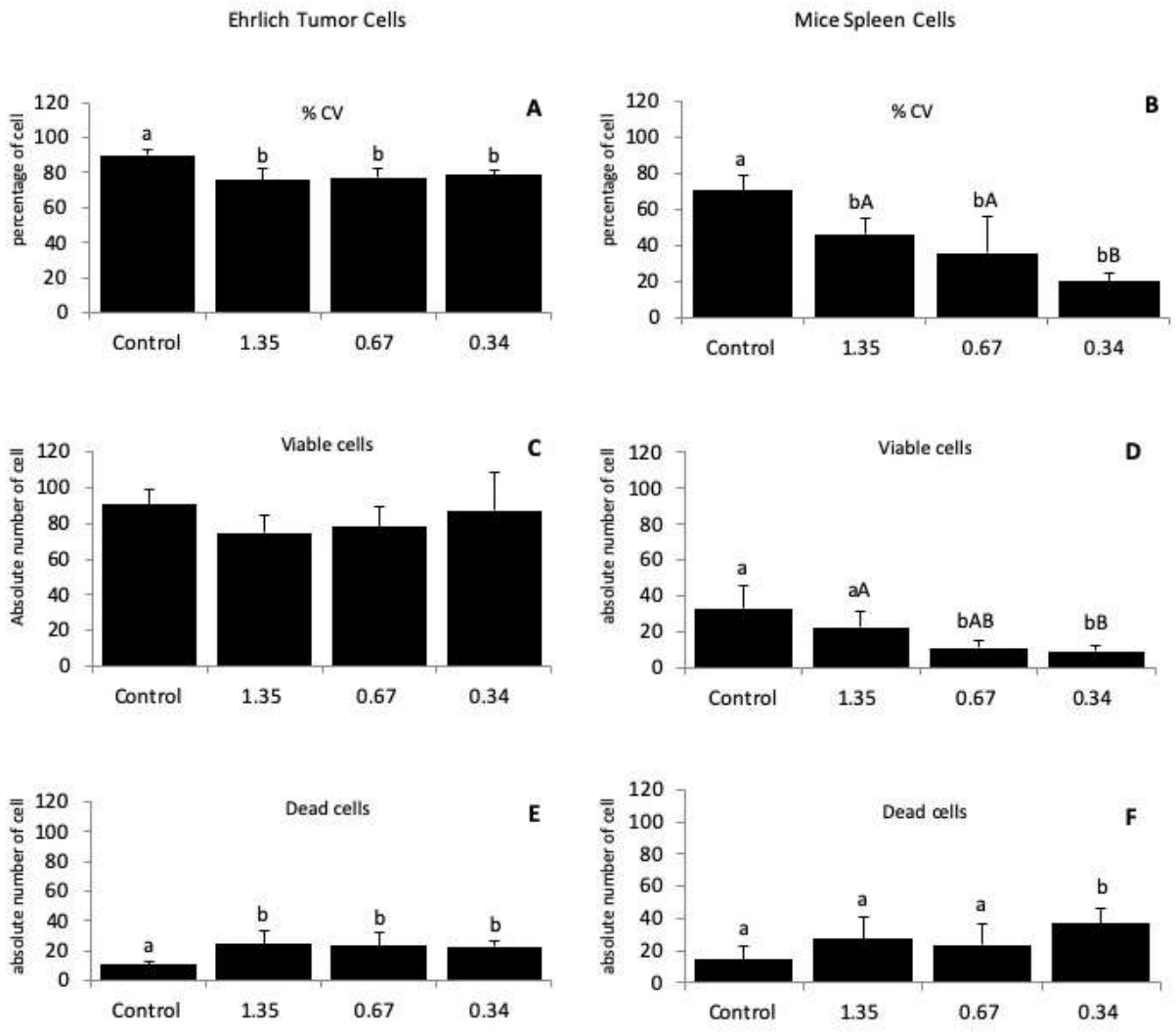

Figure 1. Percentage of Ehricih Tumor cell and mice spleen cell in co-culture (37ºC, 5% Co_2) with different dilutions of Dughetia sp ACE extract for 24 h. Values are expressed as means ± standard deviation of percentage of cell viability %CV (A and B); absolute number of viable cells (C and D); and absolute number of dead cells (E and F). The cytotoxicity assay was performed using Trypan blue exclusion method. The % CV was calculated according to the following formula: [(viable cell nº x 100) / viable cell nº + die cell nº)]. The lowercase letter denotes comparison of control and treated groups. The uppercase letter denotes comparison among treated groups.

Besides that, the cytotoxic effect of ACE and ME extracts may be higher than observed because irreversible damage to cells may not immediately lead to disrupted membrane integrity (Elia et al., 1993; Strober, 2015). In this case, it would be important to analyze how the extracts ACE and ME interfere with the regulation of the cell cycle and which type of cell death is stimulated.

Another important aspect observed was the antiproliferative effect of the ME extract for Ehrlich tumor cells, which led to a reduction in the number of viable cells at 1.4 mg mL^{-1} (Figure 2B). This antiproliferative action reflects different types of irreversible alterations in cellular function that result in cell death (Elia et al., 1993; Adan et al., 2016).

The plants of the Annonaceae family are known for their numerous bioactive compounds as such as alkaloids, terpenoids and acetogenins (Pumiputavon et al., 2017). However, few chemical data are available on the *Duguetia* genus, despite its high number of species (Almeida et al., 2012).

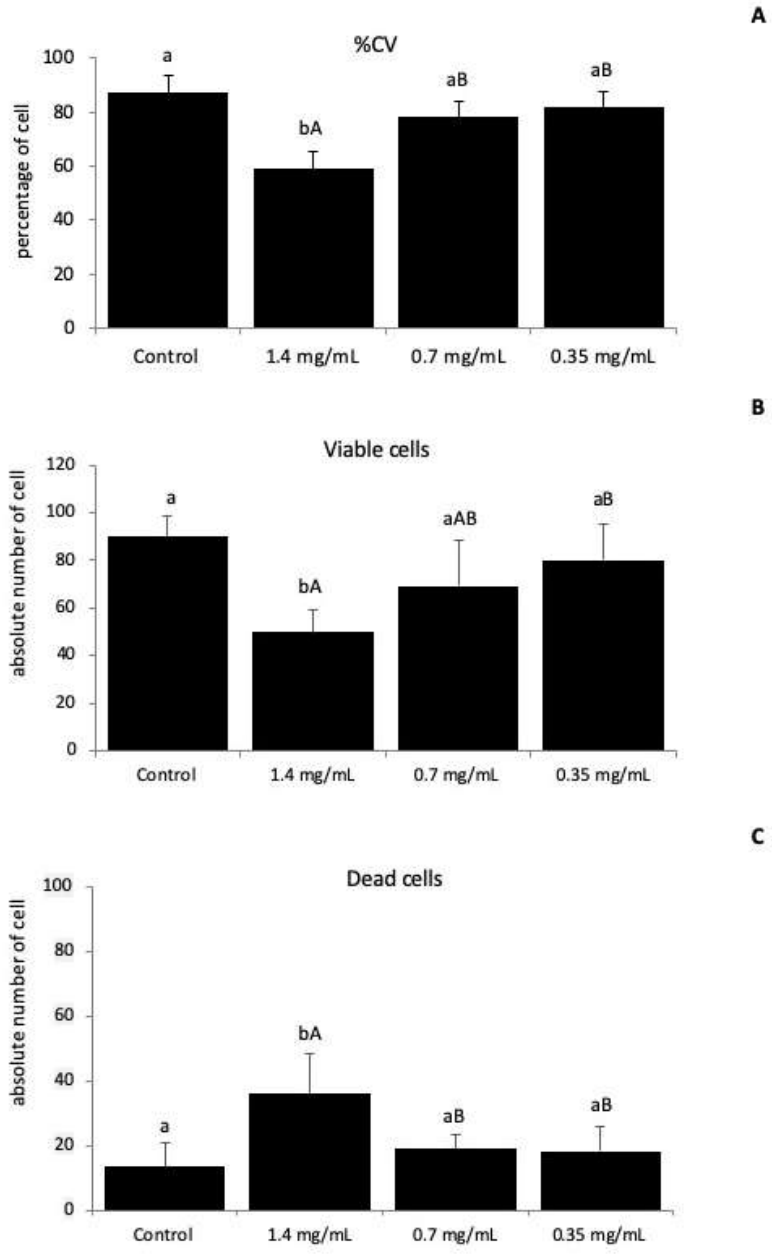

Figure 2. Percentage of Ehricih Tumor cells in co-culture (37ºC, 5%Co_2) with different dilutions of Dughetia sp ME extract for 24 h. Values are expressed as means ± standard deviation of percentage of cell viability %CV (A); absolute number of viable cells (B); and absolute number of dead cells (C). The cytotoxicity assay was performed using Trypan blue exclusion method. The % CV was calculated according to the following formula: [(viable cell nº x 100) / viable cell nº + die cell nº)]. The lowercase letter denotes comparison of control and treated groups. The uppercase letter denotes comparison among treated groups

Methanolic extracts from *D. furfuracea* leaves are rich in total phenols, flavonoids, and alkaloids (Pinho et al., 2016; Santos et al., 2018).

Flavonoids have the ability to induce apoptosis and block the cell cycle by demolishing the structure of the spindle fiber (Pumiputavon et al., 2017). Quercetin was demonstrated to be present in methanolic extract from *D. furfuracea* (Pinho et a., 2016), and this flavonoid can promote the pro-apoptotic gene Bax and activate caspase pathways (Pumiputavon et al., 2017).

Alkaloids also have cytotoxicity and anti-cancer activity (Pumiputavon et al., 2017). Anonaine, an alkaloid from *D. furfuracea*, reduces the viability of normal rat hepatocytes and HeLa tumor cells (Pérez & Cassels, 2010). For HeLa cells, the cytotoxic effect was associated with DNA damage and the blocking of the cell cycle before the G_1 phase (Pérez & Cassels, 2010). Thus, the antiproliferative and cytotoxic action observed in the ME extract may be a consequence of the alkaloids and flavonoids present in the extract.

Another important compound is acetogenin. Annonaceous acetogenins are a group of potential anti-neoplastic agents isolated from Annonaceae plants because of their strong cytotoxic activity (Yuan et al., 2006; Chen et al., 2012; Attiq et al., 2017). Their cytotoxicity is mediated by the depletion of ATP levels via the inhibition of NADH-ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport system (Zafra-Polo et al., 1998; Yuan et al., 2003; Yuan et al., 2006). Furthermore, they also inhibit the ubiquinone-linked NADH oxidase that is constitutively expressed in the membrane of cancer cells, but only transiently expressed in normal cells (Yuan et al., 2006). Using the T24 bladder transitional carcinoma cell line as a model, Yuan et al. (2003 and 2006) showed that acetogenins also arrest T24 cells in G1 phase and cause apoptotic cell death in a Bax- and caspase-3-related pathway.

ACE extract is rich in acetogenins and the presence of this compound may explain the cytotoxic effects observed in this study.

Finally, growth of mice spleen cells in the presence of ACE extract led to antiproliferative and cytotoxic effects, as was also observed in Ehrlich tumor cells. This effect was also shown by Pérez & Cassels (2010) with normal rat hepatocytes and Dutra el al. (2014) with non-tumor human peripheral blood mononuclear cells (PBMC).

Conclusion

The results of this study demonstrate that the *Duguetia* sp extracts are cytotoxic and have antiproliferative effects on Ehrlich tumor cells and mice spleen cells. These findings are important and in agreement with the results of previous studies with *Duguetia* plants, demonstrating the biological action of their compounds and confirming observations made in folk medicine. Further studies are needed to elucidate the actual species used in extract preparations, extract compounds, and mechanism of action.

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