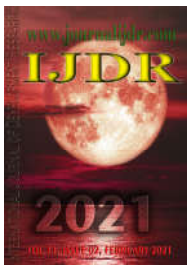
ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 11, Issue, 02, pp. 44673-44678, February, 2021
https://doi.org/10.37118/ijdr.21154.02.2021



RESEARCH ARTICLE OPEN ACCESS

ANTITUMORAL ACTIVITY OF *ELEUTHERINE PLICATA* HERB. AND ITS COMPOUNDS

Ana Laura Gadelha Castro[1], Juliana Correa-Barbosa[1], Paloma Santos de Campos[2],Bibiana Franzen Matte [2],Marcelo Lazzaron Lamers[2], José Edson de Sousa Siqueira[3],Andrey Moacir do Rosario Marinho[3], Marta Chagas Monteiro[1], Valdicley Vieira Vale[4] and Maria Fâni Dolabela[1,4,*]

[1]Postgraduate Program in Pharmaceutical Sciences, Federal University of Pará, Belém, PA, Brazil
[2]Postgraduate Program in Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[3]Postgraduate Program in Chemistry, Federal University of Pará, Belém, PA, Brazil
[4]Postgraduate Program in Pharmaceutical Innovation, Federal University of Pará, Belém, PA, Brazil

ARTICLE INFO

Article History:
Received 09th December, 2020
Received in revised form
16th December, 2020
Accepted 06th January, 2021
Published online 28th February, 2021

Key Words:
Eleutherine plicata; Isoeleutherin; Eleutherin; eleutherol; Antitumoral.

****Corresponding author:***
Maria Fâni Dolabela

ABSTRACT

The antitumoral activity of plants containing quinonic compounds has been reported, however there is a need to evaluate *Eleutherine plicata*. This study assesses the impact of *E. plicata* fractionation in its antitumoral effect. The ethanol extract obtained from *E. plicata* bulbs (EEEP) was fractionated under reflux. The dichloromethane fraction (FRDCM) was refracted in a chromatographic column, isolating eleutherol, isoeleutherin and eleutherin. The antitumoralactivity was evaluated in cell line (oral cancer-SCC-9) and normal cell line (human keratinocytes-HaCaT) using the sulforadamine, three-dimensional spheroid and cell migration assays. The greatest inhibitions of cell proliferation and selectivity index were observed with EEEP (SCC-9: IC_{50} = 12.87 ± 0.86 and HaCaT: 28.81 ± 1.82 µg/mL). Cell desagregation was influenced by the exposure time and concentration of EEEP. Greater inhibitory effect on speed and targeting was observed in tumor cells treated with EEEP. In summary, EEEP has a promisingantitumor activity.

Citation: ***Ana Laura Gadelha Castro, Juliana Correa-Barbosa, Paloma Santos de Campos, Bibiana Franzen Matte, Marcelo Lazzaron Lamers, José Edson de Sousa Siqueira, Andrey Moacir do Rosario Marinho, Marta Chagas Monteiro, Valdicley Vieira Vale and Maria Fâni Dolabela,*** **2021.** "Antitumoral activity of eleutherine plicata herb. and its compounds", *International Journal of Development Research*, 11, (02), 44673-44678.

INTRODUCTION

Eleutherine plicata (Iridaceae), known by Amazonian populations as marupazinho (Brasil, 2009), is popularly used to treat diarrhea, amoeba, intestinal infection, liver disease, hemorrhage and anemia (Pinto, 2008). Chemical studies of extracts obtained from bulbs of this plant led to the isolation of several naphthoquinones, naphthalene compounds, anthraquinones and terpenes, among the isolated constituents, the main products include naphthalene eleutherol (Figure 1 a); naphthoquinones eleutherin (Figure 1 b) and isoeleutherin (Figure 1 c) (Alves *et al.*, 2009; Paramapojn *et al.*, 2008; Malheiros *et al.*, 2015; Vale *et al.*, 2020). The antitumor activities of the ethanol extract (EEEP), dichloromentane fraction (DFEP), isoleutherin (ISO) and unidentified naphthoquinone (UN) were evaluated in hepatocarcinoma cell line (HepG2). The DFEP was cytotoxic (IC_{50} 19.04) and naphthoquinones were also cytotoxic (ISO-CI_{50} 31.11 µg/mL; NNI-CI_{50}-15.37 µg/mL). In order to verify whether the effect was related to genotoxicity, the samples were subjected to comet and micronucleus assay using the same strain. Isoeleutherin presented the lowest frequency of micronuclei (FN = 4.03%) in relation to ethanol extract (FN = 9.13%) and the dichloromethane fraction (FN = 20.43%). However, isoeleutherin showed higher cell damage index (DI = 2.07) in the comet assay, than ethanol extract (DI = 1.48), dichloromethane fraction (DI = 1.96) and negative control (DI = 0.77) (Galucio, 2014). These preliminary results may indicate that *E. plicata* has antitumor potential. The antitumoral effect of eleutherin and isoeleutherin was evaluated in different tumor lines and in the human non-tumor line HacaT (keratinocytes). The concentration that caused total cell growth inhibition (TGI), by eleutherin, in glioma, breast cancer, and HaCaT were: 2.8 µg/mL, 4.8 µg/mL and 20 µg/mL, respectively. Using the same cell lines, the eleutherol concentrations that inhibited cell growth (GI_{50}) were 3.1 µg/mL in glioma cells, 3.0 µg/mL for breast cancer, 6.3 µg/mL in leukemia and 18.7 µg/mL in keratinocytes (Campos, 2015). These results suggest a greater activity of eleutherin and isoeleutherin on tumor cells. This article describes, for the first time, the antitumoral effect of EEEP and DFEP and their activity on spheroids disaggregation and cell density as well.In addition, our search evaluated whether eleutherin, isoeleutherin and eleutherol have a greater antitumoral effect (oral squamous carcinoma of intermediate aggressiveness: SCC-9), keratinocytes (HaCaT) and compared the selective potential for tumor cells (SCC-9) with EEEP and FDEP.

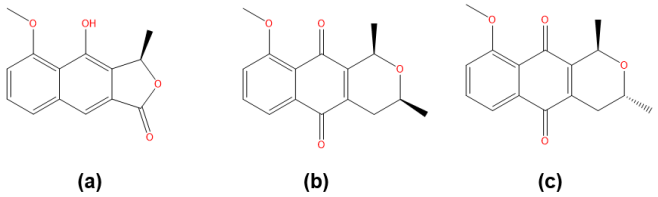


Figure 1. Substances Isolated from *Eleutherine plicata*.(a)Eleutherol; (b)Eleutherin; (c) Isoeleutherin

The antitumor activities of the ethanol extract (EEEP), dichloromentane fraction (DFEP), isoleutherin (ISO) and unidentified naphthoquinone (UN) were evaluated in hepatocarcinoma cell line (HepG2). The DFEP was cytotoxic (IC_{50} 19.04) and naphthoquinones were also cytotoxic (ISO-CI_{50} 31.11 µg/mL; NNI-CI_{50}-15.37 µg/mL). In order to verify whether the effect was related to genotoxicity, the samples were subjected to comet and micronucleus assay using the same strain. Isoeleutherin presented the lowest frequency of micronuclei (FN = 4.03%) in relation to ethanol extract (FN = 9.13%) and the dichloromethane fraction (FN = 20.43%). However, isoeleutherin showed higher cell damage index (DI = 2.07) in the comet assay, than ethanol extract (DI = 1.48), dichloromethane fraction (DI = 1.96) and negative control (DI = 0.77) (Galucio, 2014). These preliminary results may indicate that *E. plicata* has antitumor potential. The antitumoral effect of eleutherin and isoeleutherin was evaluated in different tumor lines and in the human non-tumor line HacaT (keratinocytes). The concentration that caused total cell growth inhibition (TGI), by eleutherin, in glioma, breast cancer, and HaCaT were: 2.8 µg/mL, 4.8 µg/mL and 20 µg/mL, respectively. Using the same cell lines, the eleutherol concentrations that inhibited cell growth (GI_{50}) were 3.1 µg/mL in glioma cells, 3.0 µg/mL for breast cancer, 6.3 µg/mL in leukemia and 18.7 µg/mL in keratinocytes (Campos, 2015). These results suggest a greater activity of eleutherin and isoeleutherin on tumor cells. This article describes, for the first time, the antitumoral effect of EEEP and DFEP and their activity on spheroids disaggregation and cell density as well.In addition, our search evaluated whether eleutherin, isoeleutherin and eleutherol have a greater antitumoral effect (oral squamous carcinoma of intermediate aggressiveness: SCC-9), keratinocytes (HaCaT) and compared the selective potential for tumor cells (SCC-9) with EEEP and FDEP.

MATERIALS AND METHODS

Plant material and isolation: The E. plicata bulbs were collected in Tracuateua, Pará, Brazil (Lat. 1.1436°, Long 46.9551°), an exsiccate was deposited at the Museu Paraense Emilio Goeldi (MG 202631). The ethanol extract was obtained by macerating the dry powder of the bulbs (924 g) in ethanol (2L for 7 days). The ethanol extract (44 g) was fractionated under reflux, resulting in 4 fractions n-hexane (3.69 g), dichloromethane (9.99 g), ethyl acetate (6.78 g) and methanol (21.9 g), monitored by thin layer chromatography (TLC; silica gel, Hexane: ethyl acetate 8:2). The dichloromethane fraction was refracted in a chromatographic column packed with silica gel (Sigma, 230x400 mesh, 40-63 µm) and eluted in solvents with increasing polarities (Vale et al., 2020), obtaining three isolated substances: eleutherol, isoeleutherin and eleutherin, identified by nuclear magnetic resonance (Castro *et al*., 2021).

The Nuclear magnetic resonance (NMR) spectra were measured using a Bruker Advance DPX 400 MHz NMR spectrometer (Bruker Ascend), obtaining the following spectra:

Eleutherol - ^{1}H NMR 400 MHz ($CDCl_3$): δ 1.73 (3H, d, *J*=8.0 Hz, Me-11), δ 4.10 (3H, s, OMe-10), δ 5.71 (1H, q, *J*= 8,0 Hz, H-1), δ 6.92 (1 H, d, *J*= 8.0 Hz, H-7), δ 7.39 (1 H, t, *J*= 8.0 Hz, H-6), δ 7.55 (1 H, d, *J*=8.0 Hz, H-5), δ 7.85 (1 H, s, OH-4). ^{13}C NMR 100 MHz ($CDCl_3$): δ 19.30 (C-11), δ 56.55 (C-10), δ 77.51 (C-1), δ 106.44 (C-7), δ 116.62 (C-6), δ 123.78 (C-5), δ 126.74 (C-4), δ 117.65 (C-8a), δ 126.02 (C-9a), δ 128.05 (C-4a), δ 137.35 (C-5a), δ 149.31 (C-9), δ 156.73 (C-8), δ 170.70 (C-3) (Castro et al., 2021). Eleutherin - ^{1}H NMR 400 MHz ($CDCl_3$): δ 1.36 (3 H, d, *J*= 8.0 Hz, Me-3), δ 1.53 (3 H, d, *J*= 8.0 Hz, Me-1), δ 2.19 (1 H, dq, *J*= 4.0; 16.0 Hz, H4-ax), δ 2.74 (1 H, dt, *J*= 4.0; 16.0 Hz, H-4eq), δ 3.58 (1 H, m, H-3), δ 3.99 (3 H, s, OMe-9), δ 4.85 (1 H, m, H-1), δ 7.27 (1 H, d, *J*= 8.0 Hz, H-6), δ 7.63 (1H, t, *J*= 8.0; 16 Hz, H-7), δ 7.72 (1H, d, *J*= 8.0 Hz, H-8). ^{13}C NMR 100 MHz ($CDCl_3$): δ 20.92 (Me-3), δ 21.38 (Me-1), δ 30.05 (C-4), δ 56.59 (C-9), δ 68.70 (C-3), δ 70.40 (C-1), δ 117.93 (C-8), δ 119.12 (C-7), δ 134.66 (C-6), δ 120.46 (C-4a), 134.15 (C-11a), δ 140.08 (C-5a), δ 148.83 (C-9a), δ 159.54 (C-9), δ 183.84 (C-5), δ 184.15 (C-11) (Castro *et al.*, 2021). Isoeleutherin - ^{1}H NMR 400 MHz ($CDCl_3$): δ 1.32 (3 H, d, *J*= 8.0 Hz, Me-3), δ 1.52 (3 H, d, *J*= 8.0 Hz, Me-1), δ 2.22 (1 H, dq, *J*= 4.0; 16.0 Hz, H4-ax), δ 2.68 (1 H, dd, *J*= 4.0; 16.0 Hz, H-4eq), δ 3.99 (3 H, s, OMe-9), δ 4.99 (1 H, m, H-1), δ 7.27 (1 H, d, *J*= 8.0 Hz, H-6), δ 7.63 (1H, t, *J*= 8.0; 16 Hz, H-7), δ 7.72 (1H, d, *J*= 8.0 Hz, H-8). ^{13}C NMR 100 MHz ($CDCl_3$): δ 19.93 (Me-3), δ 21.67 (Me-1), δ 29.98 (C-4), δ 56.62 (OMe-10), δ 62.64 (C-3), δ 67.58 (C-1), δ 117.99 (C-8), δ 119.28 (C-7), δ 119.93 (C-4a), δ 134.26 (C-11a), δ 134.87 (C-11a), δ 139.54 (C-5a), δ 148.23 (C-9a), δ 159.90 (C-9), δ 182.90 (C-5), δ 184.42 (C-10) (Castro *et al.*, 2021).

Assay for antitumoral activity: The cell lines HaCaT (keratinocytes - ATCC® PCS-200-011TN ^{TM}) and SCC-9 (OSCC lineages, oral squamous carcinoma of intermediate aggressiveness: SCC-9), were obtained from Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, RJ, Brazil), were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (Gibco) and glucose (Gibco), and incubated in a humid atmosphere at 37º C and 5% CO_2. When cell confluence was observed, the cells were detached with trypsin and adjusted to 10^4 cells/ml. These cells were distributed in 96-well plates (4000 cells) and after 24h, they were treated with different concentrations of EEEP, DFEP, isoeleutherin, eleutherin and eleutherol (15.62 to 700µg/mL) and then incubated for 24h. After that, the cell monolayers were fixed with 10% (v/v) trichloroacetic acid and stained with sulforhodamine B (SRB) for 30 min. The dye excess was removed by repeated washing with 1% acetic acid (v/v). The protein-bound dye was dissolved in a 10 mM tris base solution to determine the optical density (OD) at 560 nm in a multiplate reader (Vichai et al., 2006).

Three-dimensional (3D) test in spheroid model: In this assay the same cell lines (HaCaT and SCC-9) were used. The wells of a 96-well plate were coated with a solution of 0.15g of agarose + 10ml of PBS (50µL in each well), added to the cells (volume/well of 104 cells/mL), followed by incubation for 24h. After that period, under standard culture conditions, the cells condensed into single, compact spheres with ~ 100% efficiency in a three-dimensional (3D) format. Then, pictures were taken by a Zeiss microscope (10x objective). Subsequently, treatment with EEEP, DFEP samples (125, 250 and 500 µg/mL) was performed. After that, we photographed the cells in a Zeiss microscope (10x objective). The images were taken 24, 48 and 72 hours after the initial photos. The delimitation of the internal area was quantified using the program ImageJ (Friedrich et al., 2009).

Time-lapse analysis: The cells (HaCaT and SCC-9) were plated in a 6-well plate ($1x10^5$cells/well) and then treated with EEEP (15 and 250 µg/mL) for 24h. After the treatment, cells were detached with trypsin (0.25% EDTA), washed, and plated again in 6-well plates coated with collagen (5 µg/cm²) washed, in media for 3 h in incubator CO2, 5% (37 ºC). For analysis of the migration properties, phase microscopy time-lapse images were captures for a period of 24 h at 10-min intervals [migration speed and spatial trajectory (ST)] with a charge coupled device camera (Axiocam mrn, Zeiss, Göttingen, Germany) attached to an inverted microscope (Axio Observer Z1, Zeiss, Göttingen, Germany) using AXIOVISION Software (Zeiss, Göttingen, Alemanha). The values for the assessment of migration speed and ST were obtained using Image J software (National Instituteof Health, Bethesda, MA, USA) and data were processed as previously described (8) (Lamers *et al.*, 2011). For ST analysis, a polar plot graph was constructed, which represents the ST developed

by each migratory cell, where the X and Y coordinates of each trajectory were normalized to start at a virtual (X=0) and (Y=0) position.

Statistical analysisl: Statistical analysis was performed using GraphPad version 5.0 (GraphPad Software). For comparison between the groups, analysis of variance (ANOVA) was applied, followed by the Tukey post-test for proliferation and bonferroni for spheroids. The level of statistical significance was 5% ($p < 0.05$).

RESULTS

The fractionation of EEEP resulted in the DFEP, and chromatographic studies suggest the presence of quinonic compounds, (data not shown) because the absorption bands in the UV-Vis spectrum are suggestive of these compounds (247 and 270nm) (Hong *et al.*, 2008). This fraction was refracted, and the following compounds were isolated: isoleutherine, eleutherine and eleutherol. All of these samples were subjected to an antitumoral activity assessment test. The EEEP was toxic to tumor cells and keratinocytes (HaCaT), with low selectivity for tumor cells. Similar toxicity profile was observed for DFEP however, it had no selective effect for tumor cells. The pure substances presented less toxicity to tumor cells and normal cells. The isoeleutherin and eleutherin isomers were not selective and eleutherol showed low selectivity (Table 1). Regarding the antitumoral activity, we observed for all samples a direct relationship with the concentration used: the higher the concentration, the greater the inhibition of cell proliferation.The samples that most inhibited tumor cell proliferation were EEEP and DFEP. The eleutherin and eleutherol had the lowest inhibitory effects for HaCaT (Figure 2). As EEEP and FRDCM showed greater inhibitory potential against tumor cells proliferation, only these were subjected to assays with spheroids. In Figure 4, it is clear that cell disaggregation was .more accentuated in spheroids treated with the highest concentration of EEEP and FRDCM, and the increased exposure time contributed to cell disaggregation. However, a higher internal density of the spheroids was observed in the lowest concentrations and in the longest exposure times (Figures 3 and 4). A similar fact was observed in spheroids treated with FRDCM, the reduction occurred only in the lowest concentration (Figures 3 and 4). In addition to antitumor activity, the inhibitory potential of EEEP on metastases was evaluated. The EEEP reduced cellular migration of tumor cells (SCC-9), especially at a concentration of 250 µg/mL (Figure 5). However, EEEP did not interfere with HaCaT cell migration, showing selectivity for tumor cells.

DISCUSSION

The EEEP was promising in antitumor activity of SCC-9, showing selectivity for this activity. The extract fractionation led to a fraction with high levels of naphthoquinones and naphthalene, FRDCM, which was less selective for SCC-9 (Figure 2; Table 1).

Table 1. Antitumoral activity, in tumor and normal lines, by the ethanolic extract of *Eleutherine plicata*, fraction and isolated compounds and their selectivity

Sample	Cell line IC_{50}+SD (µg/mL)		Selectivity Index	Classification
	SCC09	HaCaT		
Ethanol extract	12.87± 0.86	28.81± 1.82	2.23	Low selective
Dichlorometane fraccion	23.58± 2.85	19.96± 1.22	0.84	Non selective
Eleutherol	192.70± 4.68	250.15± 5.07	1.29	Low selective
Isoeleutherin	145.43± 0.63	74.10± 2.90	0.51	Non selective
Eleutherin	166.80± 2.91	85.38± 2.04	0.51	Non selectiv

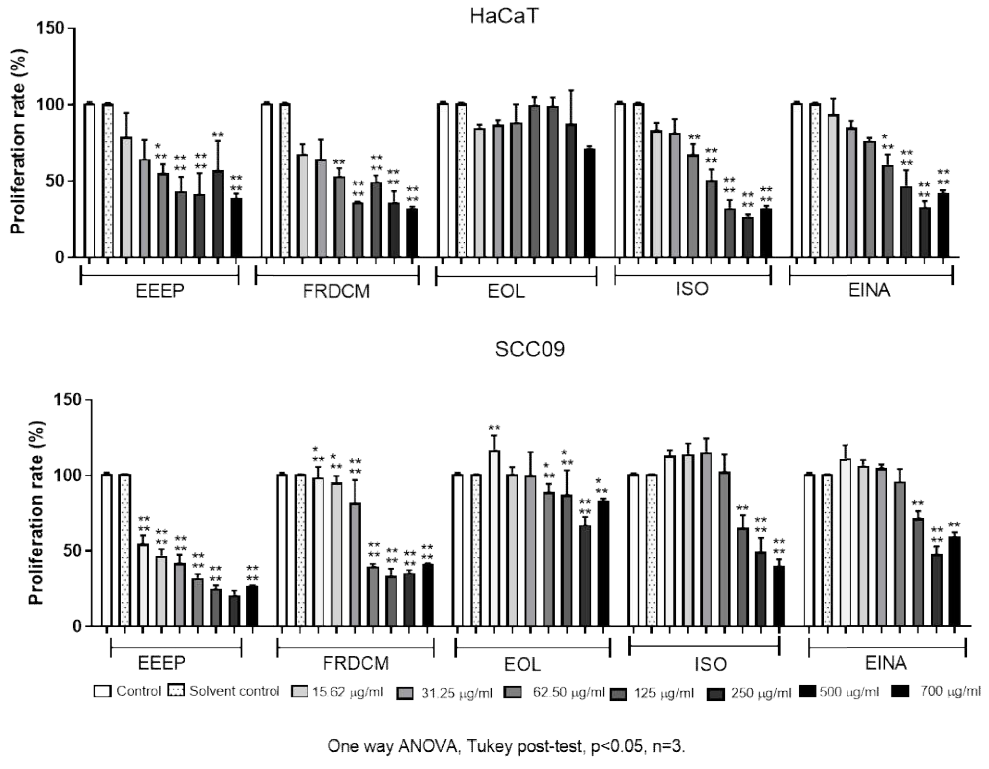


Figure 2. Antitumoral activity of *Eleutherine plicata* on tumor cell lines (SCC09) and keratinocytes (HaCaT).(EEEP) ethanol extract of *Eleutherine plicata;* (FRDCM) dichloromethane fraction of *Eleutherine plicata;* (EOL) eleutherol; (ISO) isoeleutherin; (EINA) eleutherin

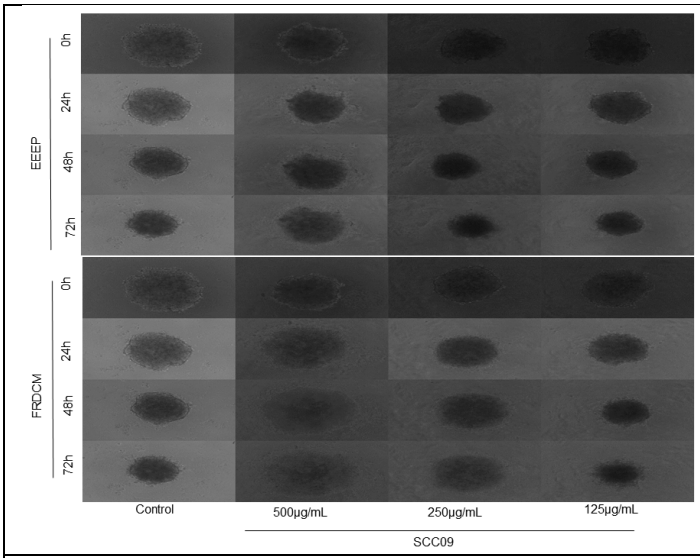


Figure 3. Images of the internal spheroid area of oral squamous carcinoma cells (SCC-9), after treatment with EEEP and FRDCM.(EEEP) ethanol extract of *Eleutherine plicata;* (FRDCM) dichloromethane fraction of *Eleutherine plicata;* The figure shows different treatment times (24, 48 and 72h) and different concentrations of EEEP and DFEP (125, 250 and 500 µg/mL)

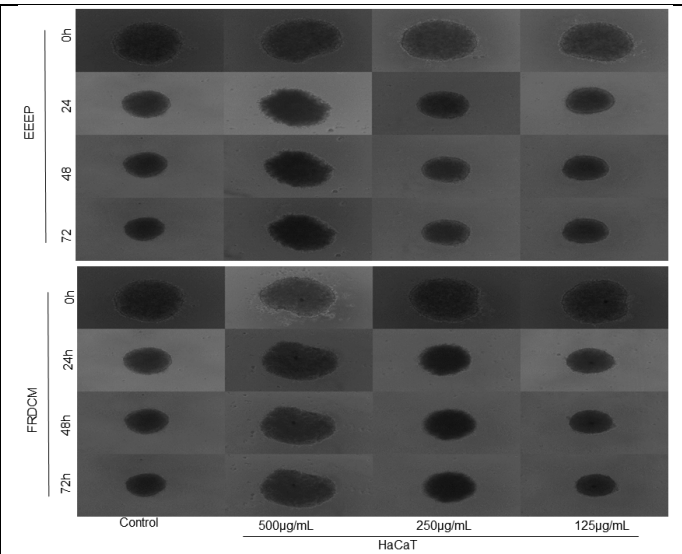


Figure 4. Images of the internal spheroid area of normal cells line of human keratinocytes (HaCaT), after treatment with EEEP and FRDCM.(EEEP) ethanol extract of *Eleutherine plicata;* (FRDCM) dichloromethane fraction of *Eleutherine plicata;* The figure shows different treatment times (24, 48 and 72h) and different concentrations of EEEP and DFEP (125, 250 and 500 µg/mL)

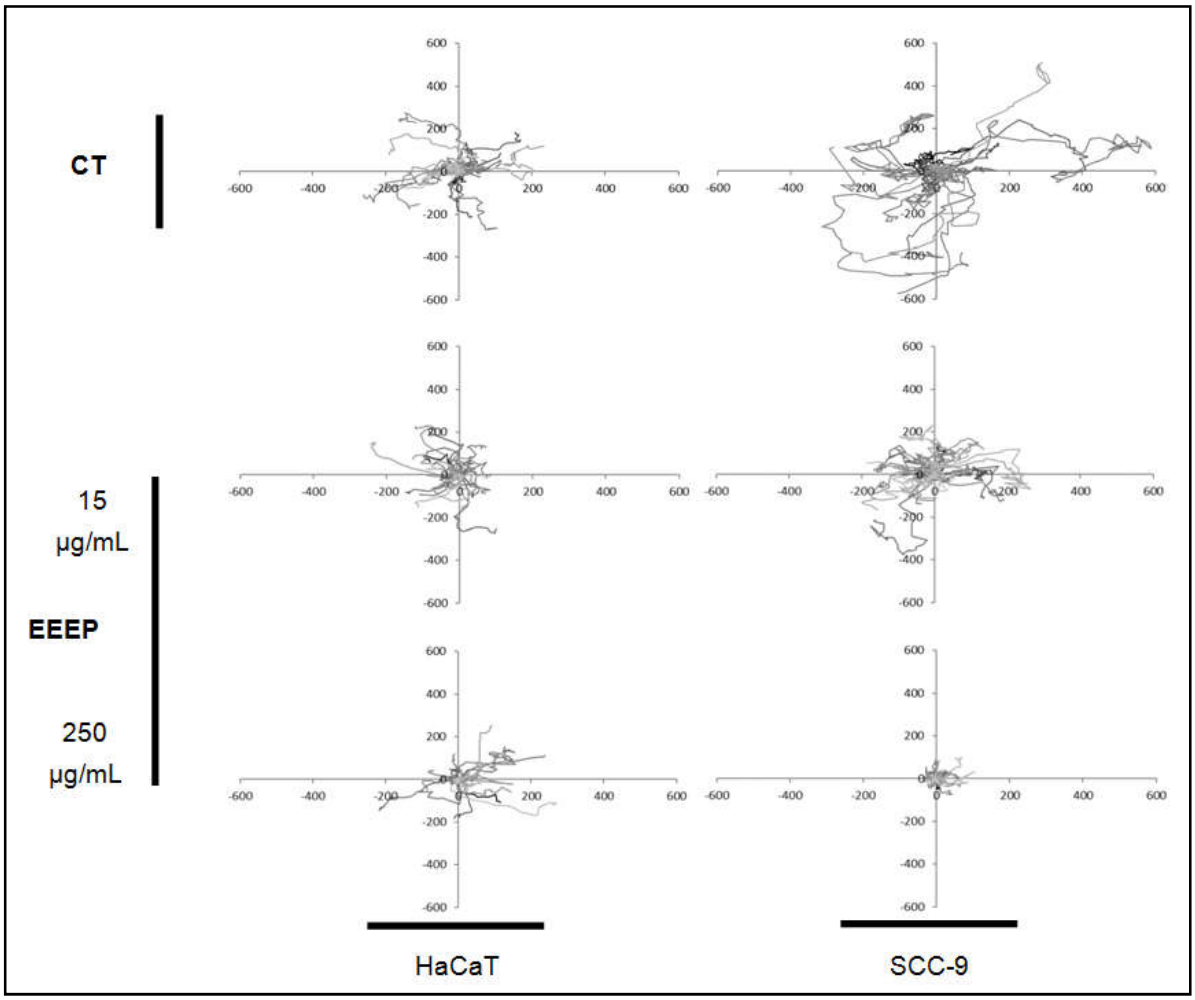


Figure 5:EEEP reduces cell migration speed and modifies directionality. TheOSCC cells (SCC-9) and no tumor cell (HaCaT) were plated in migrationpromoting conditions and imaged for 24 h (time interval, 10 min). For analysis of migration velocity (A), individual migratory cells were tracked, and the ratio of the total distance traveled by the migration time was computed. For the analysis of directionality, the spatial trajectory of each migratory cell (individual lines) from control (CT) or EEEP treatment was normalized to start at a virtual X = 0 and Y = 0. Analysis of variance, n = 3 independent experiments, *p < 0.001

This reduction in selectivity may result from synergistic effect of constituents of FRDCM. Naphthoquinones are capable of inducing oxidative stress through the intracellular formation of reactive oxygen species, such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical (HO^-), which can damage some important cellular components in both normal and malignant cells. This interference alters cell division at specific points in natural morphogenic evolution (Belizário, 2002). When altering cell normality, the cell may enter into apoptosis due to oxidative stress (Amatantes-Mendes *et al.*, 1999). Due to the generation of reactive oxygen species, cells can be rendered unviable (Machado, 2000; Silva *et al.*, 2003). Another study demonstrated that FRDCM had greater genotoxic potential than EEEP and related greater toxicity to oxidative stress (Galucio, 2014). These results corroborate the results obtained in the present study and make us suggest the use of EEEP is safer than the use of FRDCM. Another study demonstrated that fractionation contributed to the mutagenic potential, with FRDCM, isoeleuterine and eleuterine being more toxic than EEEP (Castro *et al.*, 2021). These results strengthen the thesis that EEEP can be used for medicinal purposes.

The isolation of naphthoquinones, eleutehrin and isoeleutherin, led to a reduction in antiproliferative activity in SCC-9 and HaCaT compared to EEEP and FRDCM (Figure 2, Table 1). Also, there was a reduction in selectivity when compared to EEEP. These results reinforce the hypothesis that the inhibition of cell proliferation by EEEP and FRDCM resulted from a synergistic effect between compounds. Studies have shown that structural changes in naphthoquinones can interfere in their anti-tumor potential and other activities. Aiming to understand the antitumor effects of naphthopyran derivatives (eleutherin, isoeleutherin and eleutheriol), a study evaluated immune responses mediated by T helper cells. This study revealed that isoeleutherin, which has a 1,4-naphthoquinone ring with α-methyl group, selectively and specifically stimulated the production of IFNc by activating the transcription of the T-bet gene, thus increasing Th1-mediated immune responses. However, a natural naftopiran-4-one, eleutherinol, dramatically inhibited the production of IFNc and IL-2 during Th cell activation, suppressing the cytokine gene transcripts. Therefore, chemical modification and chirality of the naphthopyran portion in isoeleutherin and eleutherinol may be critical for selective modulation of immune responses mediated by T helper cells and in the antitumor response (Hong *et al.*, 2008). Initially, we expected isoeleutherin would inhibit cell proliferation of SCC-9, but this did not occur. Another study evaluated the activity of inhibiting cell proliferation, in different tumor cell lines, by isoeleutherin and eleutherin, with a promising response (Campos, 2015). It is worth mentioning that different tumor strains have different sensitivity to drugs (ATCC, 2020; Matuo *et al.*, 2011), It may justify the different responses obtained. The *in vitro* model for studying classic anticancer drugs is the monolayer cell culture. The two-dimensional (2D) model can make significant contributions to cancer research, another important model for studying the activity of molecules in cancer is the three-dimensional (3D) culture model. Comparing the two models, 3D culture can provide a microenvironment that more reliably mimics the tumor tissues microenvironment (Wen *et al.*, 2013).

In this context, samples effect on multicellular spheroids was evaluated. The greatest cell breakdowns were observed at the highest doses and the longest exposure times of cells (SCC-9 and HaCaT) to EEEP and FRDCM (Figures 3.4). The molecular bases of the cell disaggregation process may involve the rupture of intercellular junctions, with consequent development of mesenchymal epithelium transition that lead to an increase in migratory, invasive and metastatic potential (INCA, 2020). The EEEP affected cell-cell adhesion of the tumor at 250 µg/mL and 500 µg/mL concentrations after 48h, while FRDCM affected cell-cell adhesion at the same concentrations, after 24h. In both samples, at a concentration of 125 µg/mL, the multicellular spheroid behaved similarly to the control, and remained very cohesive (Figure 3). The keratinocyte culture was little affected, there were changes in the dosages of 500 µg/mL in both samples, and in 250 µg/mL in FRDCM, the dispersion in this strain was very succinct (Figure 4). The 3D assay is an environment that best mimics the tumor, so there is greater difficulty for the substance to diffuse through the tumor. In the present study, the EEEP and FRDCM penetrate the tumor by breaking cell-cell adhesions, further increasing the tumor permeability. It was interesting that *E. plicata* caused a greater dissociation of spheroid cells in the tumor cell line, but little affected keratinocytes, which may indicate, a selectivity, and an important therapeutic potential. Despite being promising as an anti-tumor, the EEEP still had a knowledge gap regarding its inhibitory potential on metastasis. When talking about metastases, the most critical steps are: cell migration and invasion. It is known that metastatic cells are highly migratory and invasive due to their cytoskeleton being poorly structured, in addition to little adhesion to its substrate (Ortíz-López *et al.*, 2009). EEEP decreases the directionality of cell migration, migration trajectories of SCC9 cells treated 250 µg / mL. In addition, the analysis of the directionality of the normal lineage cell showed that the EEEP had little impact on the directionality (Figure 5), suggesting a selectivity for tumor lineage. The reduction in directionality may be related to inhibition / decrease of proteins responsible for cell migration (Lin *et al.*, 2010). In the case of cancer with high metastatic potential, the inclusion of compounds that inhibit cell migration, reducing the possibility of metastasis, can contribute to the increase in the effectiveness of chemotherapy (Almeida *et al.*, 2005). This benefit can be even greater if there is selectivity for tumor cells (Cragg*et al.*, 2005). EEEP 2 appears to be selective for the tumor lineage, interfering with very little migration speed and directionality in normal cells.In summary, the study demonstrated the anti-tumor potential of EEEP and FRDCM. The results revealed the isolation of isoeleutherine and eleutherine naphthoquinones does not contribute significantly to the activity, suggesting that the antitumor effect results from synergism between the constituents. In spheroid studies, we observed that two factors can interfere with the antitumoral response: the exposure time and the concentration used. However, the time of exposure and concentration had little influence on cytotoxicity in normal cells. In addition, EEEP selectively inhibited the migration of tumor cells, reaffirming that E. plicata has an anti-tumor potential. It is believed that this activity is related to synergisms between the chemical constituents of the species.

Acknowledgments

This study was developed with support from the Programa Nacional de Cooperação Acadêmica na Amazônia- Procad/Amazônia of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Capes/Brazil and Edital Universal 2018-CNPq (Process 432458/2018-2).We thank Dr. Marlia Regina Coelho Ferreira (Museu Paraense Emílio Goeldi) for the identification of the plant material and the deposit of the exsiccatae at the Herbário João Murça Pires; the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the funding.

Declarations of Competing interest: The authors declare that there is no conflict of interest. Financing agencies had no role in the design of the project; analysis or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

REFERENCES

Almeida VL, Leitão A, Reinai LCB, Montanarii CA, Donnicii CL, Lopesi MTP (2005). Câncer e agentes antineoplásicos ciclo-celular específicos e ciclo-celular não específicos que interagem com o DNA: uma introdução. Química Nova. 28(1): 118-129.

Alves, TMA, Kloos H, Zani CL (2003). Eleutherinone, a novel fungitoxic naphthoquinone from *Eleutherine bulbosa* (Iridaceae). Mem Inst Oswaldo Cruz. 98, 709-712. https://doi.org/10.1590/S0074-02762003000500021.

Amarantes-Mendes GP, Green DR (1999).The regulation of apoptotic cell death. Braz J Med Biol Res.*32:* 1053-1061.

Belizário JE (2002). Reverter o cancer: o próximo desafio. Ciência Hoje. 31, 50-57.

Brasil(2009). Renisus, Relação Nacional de Plantas Medicinais de interesse do SUS.http://bvsms.saude.gov.br/bvs/sus/pdf/marco/ms_relacao_plantas_medicinais_sus_0603.pdf (accessed 17 September 2020).

Campos A(2015). Análise fitoquímica e avaliação da atividade antiproliferativa de espécies adaptadas da flora Catarinense: *Synadenium grantii, Cipura paludosa, Epidendrum mosenii* e *Maytenus robusta.* Tese de doutorado, Universidade do Vale do Itajaí, Santa Catarina.

Castro ALG, Cruz JN, Sodre DF, Correa-Barbosa J, Azonsivo R, Oliveiria MS, Siqueira JES, Galucio NCR, Bahia MO, Burbano RMR, Marinho AMR, Percario S, Dolabela MF, Vale VV (2021). Evaluation of the Genotoxicity and Mutagenicity of Isoeleutherin and Eleutherin Isolated from *Eleutherine plicata* Herb. Using Bioassays and *In silico* Approaches. Arabian Journal of Chemstry.*In process*.

Cragg GM, Newman DJ (2007). Plants as a source of anti-cancer agents. Journal Of Ethnopharmacology. 100(1-2): 72-79.

Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA (2009). Spheroid-based drug screen: considerations and practical approach. Nature Protocols. (4): 309-324. https://doi.org/10.1038/nprot.2008.226.

Galucio NCR (2014). Estudos Fitoquímicos, Citotoxicidade e Genotoxicidade de *Eleutherine plicata* Herb.Master's thesis in pharmaceutical sciences, Universidade Federal do Pará, Belém.

Hong J-H, Yu ES, Han AR, Nam JW, Seo EK, Hwang ES(2008). Isoeleutherin and eleutherinol, naturally occurring selective modulators of Th cell-mediated immune responses. Biochemical and Biophysical Research Communications. 371: 278-282. https://doi.org/10.1016/j.bbrc.2008.04.060.

Instituto Nacional do Câncer. Oncobiologia celular e molecular. https://www.inca.gov.br/pesquisa/pesquisa-experimental-e-translacional/oncobiologia-celular-e-molecular (accessed 9 September 2020).

Lamers ML, High glucose-mediated oxidative stress impairs cell migration. PLoS One. 6(8): e22865,2011.

Lin CW, Shen SC, Chih CC, Yang LY, Shia LT, Chen YC (2010). 12-*O*-tetradecanoylphorbol-13-acetate-induced invasion/migration of glioblastoma cells through activating PKCα/ERK/NF-κB-dependent MMP-9 expression. Journal of Cellular Physiology. 225(2): 472-481.

Machado AEH(2000).Terapia Fotodinâmica: Princípios, Potencial de Aplicação e Perspectivas.Quim. Nova.23: 237-243.

Malheiros LCS, Mello JCP, Barbosa WLR (2015). *Eleutherine plicata* – quinones and antioxidante activity, in:Phytochemicals – isolation, characterisation and Role in Human Health. Rao, V.; Rao, L. (Eds.), Intech Open: Londres, Inglaterra, 323-338.

Matuo MCS (2011). Indução do sistema citocromo P450 em linhagem dehepatoma humano para à utilização como modelo *in vitro* para aodesenvolvimento de fármacos. Ph. D. Thesis in pharmaceutical sciences, Universidade de São Paulo,São Paulo.

Organização mundial de recursos e padrões de materiais biológicos. ATCC, Cell lines. https://www.atcc.org/Products/Cells_and_Microorganisms/Cell_Lines.aspx (accessed 17 September 2020).

Ortíz-Lopez L, Morales-Mulia S, Ramírez-Rodríguez G, Benítez-King G (2009). ROCK-regulated cytoskeletal dynamics participate in the inhibitory effect of melatonin on cancer cell migration. *Journal of Pineal Research*. 46(1): 15-21.

Paramapojn S, Ganzera M, Gritsanapan W, Stuppner H (2008). Analysis of naphthoquinone derivatives in the Asian medicinal plant *Eleutherine americana* by RP-HPLC and LC-MS. J Pharm Biomed Anal. 47: 990-993. https://doi.org/10.1016/j.jpba.2008.04.005.

PintoLN(2008). Plantas medicinais utilizadas por comunidades do município de Igarapé Miri-Pará:etnofarmácia do município de Igarapé Miri. Master's thesis in pharmaceutical sciences, Universidade Federal do Pará, Belém.

Silva MN, Ferreira VF, Souza MCBV (2003). Um panorama atual da química e da farmacologia de naftoquinonas, com ênfase na beta-lapachona e derivados.Quím. Nova. 26: 407-416. https://doi.org/10.1590/S0100-40422003000300019.

Vale VV, Cruz JN, Viana GMR, Póvoa MM,Brasil DSB, Dolabela MF(2020). Naphthoquinones isolated from *Eleutherine plicata* herb: *in vitro* antimalarial activity and molecular modeling to investigate their binding modes. MedicinalChemistry Research. 29: 487-494. https://doi.org/10.1007/s00044-019-02498-z.

Vichai V, Kirtikara K(2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature Protocols. 1: 1112-1116. https://doi.org/10.1038/nprot.2006.179.

Wen Z, Liao Q, Hu Y, You L, Zhou L, Zhao Y (2013). A spheroid-based 3-D culture model for pancreatic cancer drug testing, using the acid phosphatase assay.Braz J Med Biol Res. 46: 634-642. https://doi.org/10.1590/1414-431X20132647.

****