

Investigation of the Possible Antioxidant and Anticancer Effects of *Croton argyrophyllus* (Euphorbiaceae)

Ricardo Guimarães Amaral^{a,*}, Luciana Nalone Andrade^c, Patrícia Severino^c, Silvan Silva de Araújo^a, Matheus Ismerim Silva Santos^a, Antônio Santos Dias^a, Manoel Odorico de Moraes Filho^b, Cláudia do Ó Pessoa^b, Adriana Andrade Carvalho^a, Sara Maria Thomazzi^a, Charles dos Santos Estevam^a

^a Department of Physiology, Federal University of Sergipe, CEP 49100-000 São Cristóvão, Sergipe, Brazil

^b Department of Physiology and Pharmacology, Federal University of Ceará, CEP 60430-270 Fortaleza, Ceará, Brazil

^c Laboratory of Nanotechnology and Nanomedicine, University Tiradentes, CEP 49032-490 Aracaju, Sergipe, Brazil

The aim of this study was to investigate the possible antioxidant and anticancer activities of the essential oil from the leaves of *Croton argyrophyllus* Kunth (EOCA). In order to evaluate the antioxidant effect, two tests were performed: 1) *in vitro* lipid peroxidation activity, at the concentrations of 50, 100 and 200 µg/mL, with a reduction in malondialdehyde (MDA) values of 4.26 ± 0.15 , 2.92 ± 0.16 and 1.61 ± 0.27 nmol EqMDA/mL, respectively; 2) activity against DPPH, at concentrations between 50 and 150 µg/mL, with IC_{50} 187.61 µg/mL. For the evaluation of a possible anticancer activity of EOCA, the *in vitro* cytotoxic activity was first determined on cultured tumor cells, showing IC_{50} values of 14.81 µg/mL, 21.86 µg/mL and 32.79 µg/mL against SF-295, OVCAR-8, and MDA/MB-435, respectively. For tumor cell lines HCT-8 and HL-60, the IC_{50} values were > 50 µg/mL for both cells. Besides those, hemolytic assay, evaluation of *in vivo* tumor growth and systemic toxicological evaluation were performed, but no significant statistical change was observed. In conclusion, the EOCA has antioxidant activity and anticancer activity *in vitro* against cancer cell lines tested without *in vivo* antitumor activity.

1. Introduction

Croton is a genus of the Euphorbiaceae, which comprises more than 1300 species, that grow in the tropical and subtropical regions of the world (Berry et al., 2005). There are some species of this genus that present antioxidant and anticancer activity as *C. bonplandianus* (Qaisar et al., 2013; Bhavana et al., 2016), *C. lechleri* (De Marino et al., 2008; Salatino et al., 2007) and *C. zambesicus* (Aderogba et al., 2011; Salatino et al., 2007). Many members of *Croton* genus are aromatic and produce essential oils on distillation, which are used for medicinal purposes such as inflammations, gastric ulcers, diabetes, diarrheas, rheumatism, wound healing, cancer and as anti-inflammatory and analgesic agents (Compagnone et al., 2010). Bioactive natural products play important roles in modern drug development, especially anticancer agents. It has been widely reported that various pharmacological activities of such compounds are related to their antioxidant properties (Bezerra et al., 2016).

Croton argyrophyllus Kunth, commonly known as “velame falso” or “marmeleiro” is found in South American countries such as Brazil, Colombia, Bolivia, and Venezuela, with wide distribution across Caatinga and Cerrado where it possesses ethnobotanical use in the treatment of heart diseases and as tranquilizer (Cruz et al., 2017). Although there is a wide variety of data on antioxidant activity and anticancer activity in other *Croton* species, knowledge about *C. argyrophyllus* anticancer activity is non-existent.

Based on the considerations above, we decided to investigate the effects of the essential oil of *Croton argyrophyllus* leaves (EOCA), assessing of the possible antioxidant and anticancer actions, as well as the systemic toxicity of the EOCA.

2. Plant material and extraction of the essential oil

Fresh leaves (1750 g) were collected in the city of the semiarid region, called Olho D'Água do Casado, Alagoas state, Brazil. The plant was identified and a sample was deposited under the number 22792 ASE. The leaves were subjected to hydrodistillation for 3 hours in a Clevenger apparatus. Subsequently, the oil was collected and dried with anhydrous sodium sulfate and stored at - 4°C until analysis following the methodology described by Sethuraman (2011).

3. Evaluation of the antioxidant activity of the EOCA

3.1 *In-vitro* lipid peroxidation assay

The potential of the EOCA to reduce lipid peroxidation was measured using the production of thiobarbituric acid-reactive substances (TBARS) through the standard assay. Briefly, egg yolk homogenate (1% w/v, 1 mL) in phosphate buffer (pH 7.4) was treated with 0.1 mL ferrous sulphate (FeSO₄, 0.17 mol/L), in the presence and absence of different concentrations of the EOCA (50, 100 and 200 µg/mL). Trolox (standard antioxidant) was used as positive control (PC, 50 µg/mL) and water as negative control. The mixture was incubated at 37°C for 30 min. Upon cooling, samples (0.5 mL) were centrifuged with 15% trichloroacetic acid (TCA, 0.5 mL) at 1200 rpm for 10 min. Supernatant was taken (0.5 mL), mixed with 0.67% thiobarbituric acid (TBA, 0.5 mL), incubated at 95°C for 60 min. After cooling, the formation of TBARS was measured by reading the supernatant absorbance at 532 nm and the result expressed as malondialdehyde equivalents (Eq MDA) of the substrate.

3.2 Assay against free radical DPPH

The antioxidant activity was determined by the ability of the antioxidants present in the oil to scavenge the stable radical DPPH (Aksoy et al., 2013). A 0.1 µM DPPH methanol solution was prepared in order to provide absorbance at 517 nm between 0.9 and 1.1. The determinations were performed by adding EOCA dissolved in methanol at DPPH solution in each well of the microplate that was sufficient to achieve concentrations from 50, 70, 90, 110, 130 and 150 µg/mL in a final volume of 290 µL. In the same proportions, methanol or butylated hydroxytoluene (BHT, 0 - 6 µg/mL) were added to the negative and positive controls, respectively. The absorbance readings were performed after 30 min of reaction in a microplate reader (ELISA) with incubation at 25°C. The IC₅₀ values were calculated through linear regression ($r^2 = 0.965$) of plots, where x-axis represented the concentration (µg/mL) and y-axis represented the scavenging effect (% inhibition) at time 30 minutes.

4. Evaluation of the possible anticancer activity of EOCA

4.1 Evaluation of the anticancer activity *in vitro*

The cytotoxicity of EOCA was evaluated using five human cell lines SF-295 (Glioblastoma), OVCAR-8 (ovarian adenocarcinoma), MDA/MB-435 (Melanome), HCT-8 (colon) and HL-60 (leukemia). The general viability of cultured cells was determined by the reduction of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product, as previously described by Mosmann (1983). For all experiments, cells were seeded in 96-well plates 0.1×10^5 cells/mL (SF-295, OVCAR-8, MDA/MB-435 and HL-60) and 0.7×10^5 cells/mL (HCT-8). After 24 h, the EOCA, dissolved in 0.7% DMSO, was added to each well. Then, the cells were incubated for 72 h at 37°C in a 5% CO₂ atmosphere. The experiment was performed as three independent experiments, using DMSO at 1% and doxorubicin at 1 µg/mL, as negative and positive controls, respectively. At the end of the incubation, the plates were centrifuged, and the medium was replaced with fresh medium (150 µL) containing 0.5 mg/mL MTT. Three hours later, the formazan product was dissolved in 150 µL DMSO, and absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter Inc.).

4.2 Hemolytic assay

The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mm CaCl₂, following the method as described by Andrade (2015). Concentrations of EOCA (0–1000 µg/mL) were added to the suspension of red blood cells obtained from six mice according to methodology adapted from Pita (2012). The tubes with the essential oil erythrocyte mixtures were incubated on a mixer for 60 min and then centrifuged at 3000 rpm for 5 min. Mixtures were incubated on a mixer for 60 min and then centrifuged at 3000 rpm for 5 min. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the hemoglobin released was measured spectrophotometrically as the absorbance at 540 nm.

4.3 Evaluation of the anticancer activity *in vivo*

Ten-day-old sarcoma 180 ascites tumor cells (2×10^6 cell/500 μL) were implanted subcutaneously into the right axillary region of experimental mice (swiss, female). One day after inoculation, the mice were separated into 6 groups (n=10 animals/group) and submitted to treatment for 7 days intraperitoneally (i.p.). Group 1: administration 10% Dimethyl sulfoxide (DMSO), negative control; Group 2: administration of 5-fluorouracil 25 mg/kg/day (5-FU), positive control; Group 3: administration of EOCA 25 mg/kg/day; Group 4: administration of EOCA 50 mg/kg/day; Group 5: administration of EOCA 100 mg/kg/day; Group 6: administration of EOCA 150 mg/kg/day. On day 8, under 1.5% isoflurane inhalation anesthesia, the mice were sacrificed and the tumors were excised and weighed. The inhibition ratio (%) was calculated through the following formula: inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the average tumor weight of the negative control, and B is the tumor weight of the treated group. The Animal Studies Committee of the Federal University of Sergipe approved the experimental protocol (CEPA/UFS 44/2012).

4.4 Systemic toxicological evaluation

Variation in body mass, change in mass of organs and total leukocyte counts were determined in all animals subjected to evaluation for tumor growth *in vivo*. For the evaluation of variation in body mass, the mice were weighed at the start and end of the experiment. Peripheral blood samples of the mice were collected and the animals were sacrificed by cervical dislocation. After sacrifice, the liver, spleens and kidney were removed and weighed. The wet mass of each organ was expressed as grams per 100 g of body mass and compared with the vehicle group. For the hematological analysis, total leukocyte counts were determined by standard manual procedures using peripheral blood samples (Amaral et al., 2015).

5. Results and discussion

5.1 *In-vitro* lipid peroxidation inhibitory activity

In this study, the EOCA at all concentrations examined, showed antioxidant performance, minimizing the product formation generated by lipid peroxidation, the MDA (nmol EqMDA/mL) when compared with negative control ($p < 0.05$). At concentrations of 50, 100 and 200 $\mu\text{g/mL}$ the EOCA showed MDA values of 4.26 ± 0.15 , 2.92 ± 0.16 and 1.61 ± 0.27 nmol EqMDA/mL, respectively. Negative control and positive control showed MDA values of the 4.81 ± 0.29 and 0.23 ± 0.01 nmol EqMDA/mL, respectively, as it can be seen in Figure 1. Some species of *Croton*, such as *C. nepetaefolius*, *C. zehntneri* and *C. argyrophyllus*, collected in Viçosa city (Ceará-Brazil), showed antioxidant activity in the TBARS model, according to the authors, due to arylpropanoids, monoterpenes and sesquiterpenes present in its composition (Morais et al., 2006). According to Araujo et al. (2014) and Ramos et al. (2013) the EOCA also presents in its constitution monoterpenes and sesquiterpenes what would justify the reduction of the formation of MDA in the evaluated concentrations. In addition, Ramos et al. (2013) also demonstrates the antioxidant capacity of EOCA through lipoperoxidation assay and confirms data found in the present work. This performance demonstrates the ability to inhibit the initiation of Fenton reaction, which is based on the transfer of electrons between the hydrogen peroxide (H_2O_2) and the ion (Fe^{2+}) acting as a homogeneous catalyst (Britto and Rangel, 2008).

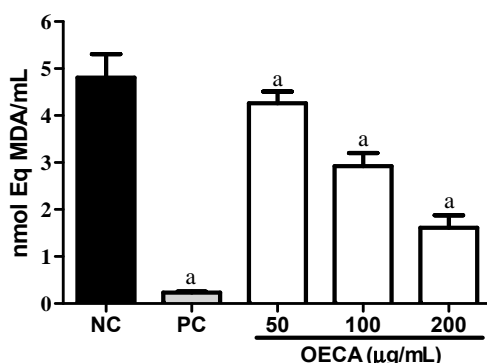


Figure 1. Trolox is the positive control (PC) and the negative control (NC) is water. Data are presented as mean \pm SEM. ^a $p < 0.05$ when compared with NC. One-way ANOVA followed by Bonferroni post-hoc test was applied.

5.2 Antioxidant activity against free radical DPPH

The test with free DPPH is considered as the model that mostly approaches the biological oxidative reactions (Afoulous et al., 2011). Aiming to evaluate the EOCA ability to capture free radicals, the DPPH test was performed. In order to investigate the potency of EOCA against free radical, the IC₅₀ and this confidence interval were determined. The results are shown in Table 1.

Table 1: Evaluation of antioxidant activity EOCA against free radical (DPPH)

	BHT (µg/mL)	<i>Croton argyrophyllus</i> (µg/mL)
IC ₅₀	3.99	187.61
CI95%	3.91 – 4.08	161.5 – 213.7

Data are presented as IC₅₀ values in µg/mL and their 95% confidence interval (CI95%) from three independent experiments, performed in quadruplicate, after 30 min of reaction.

These results allow us to affirm that the EOCA did not obtain satisfactory performance against DPPH. Since, according to Melo et al. (2010), complex samples, which exhibit IC₅₀ above 140 µg/mL are considered to have low antioxidant activity.

5.3 Anticancer activity *in vitro* of the EOCA

It has been seen that most natural products with antioxidant activity either act as anticancer agents or prevent cellular damage, what gives them a significant importance for the human health (Nath et al., 2013). Thus, we decided to assess the anticancer activity *in vitro* of the EOCA against several human tumor cell lines. The *in vitro* assay values were expressed as half-maximal inhibitory concentration (IC₅₀) and are shown in Table 2.

Table 2: Anticancer activity *in vitro* of the EOCA on human cancer cell lines.

Cell lines	Histotypes	Doxorubicin µg/mL	<i>Croton argyrophyllus</i> µg/mL
SF-295	Glioblastoma	0.20 0.16 – 0.33	14.81 11.37 – 19.29
OVCAR-8	Ovarian	0.90 0.77 – 1.50	21.86 19.29 – 24.77
MDA/MB-435	Melanoma	0.50 0.36 – 0.59	32.79 30.1 – 44.3
HCT-8	Colon	0.01 0.01 – 0.03	> 50
HL-60	Leukemia	0.02 0.01 – 0.02	> 50

Data are presented as IC₅₀ values and their 95% confidence interval from three independent experiments, performed in triplicate, and measured through the MTT assay. Doxorubicin was used as positive control.

Cytotoxicity through the MTT analysis method has been used in screening the National Cancer Institute program from the United States (NCI), testing over 10.000 samples every year (Skehan et al., 1990). The EOCA displayed anticancer activity against all tumor cell lines tested. It showed IC₅₀ values lower than 30 µg/mL against SF-295 and OVCAR-8. For the front tumor cell line MDA/MB-435, HCT-8, and HL-60, the IC₅₀ values were > 30 µg/mL for all three cells. According to the preclinical anticancer drug screening program used in this study, an essential oil that shows IC₅₀ values below 30 µg/mL is considered promising (Ferraz et al., 2013). Therefore, we considered that the EOCA presents potent anticancer activity *in vitro* against SF-295 and OVCAR-8 and weak anticancer activity *in vitro* against MDA/MB-435, HCT-8, and HL-60.

In the work performed by Ramos et al. (2013) and Araujo et al. (2014), it was observed that the EOCA has the bicyclogermacrene as a major constituent. The bicyclogermacrene has been described with weak cytotoxic activity against human breast cancer cells (MCF-7) (Afoulous et al., 2011). Several minority constituents of the essential oil of *Croton argyrophyllus* described by Ramos et al. (2013) have been described in the literature for their cytotoxic activity on tumor cell lines as δ-elemene (WANG et al., 2006), β-elemene (Dutra et al., 2012), caryophyllene oxide (Sibanda et al., 2004), α-humulene (El Hadri et al., 2010), mircenol and α-pinene (Sobral

et al., 2014). Then, probably, the anticancer activity *in vitro* of the EOCA tested might be attributed to the synergic effects of some of its constituents.

5.4 Hemolytic assay

Since EOCA showed cytotoxicity in tumor cells, it was tested for its ability to induce lysis mouse erythrocytes (data not shown). However, EOCA was not hemolytic even at the highest concentration tested (1000 µg/mL). That suggests that the cytotoxicity mechanism is probably related to a more specific pathway.

5.5 Evaluation of anticancer activity *in vivo*

The next step of this study was the evaluation of the *in vivo* anticancer activity of the EOCA. One day after the end of the treatment, the average tumor weight of the negative control mice was 1.83 ± 0.13 g. In the presence of the EOCA (50, 75, 100 and 150 mg/kg/day), the average tumor weights were 1.79 ± 0.15 g, 1.56 ± 0.10 g, 1.61 ± 0.15 g, 1.59 ± 0.13 g, respectively. Nevertheless, there was no statistically significant difference when we compared groups treated with EOCA with negative control group. Although some of the EOCA constituents have cytotoxic activity against tumor cell lines, the constituents and EOCA were never tested against Sarcoma 180 cells. It is therefore possible that EOCA is not cytotoxic to Sarcoma 180 cells, which would justify the non-tumor activity *in vivo* at the doses tested.

5.6 Systemic toxicological evaluation

Henceforth, toxicological parameters in mice with tumor and treated with the EOCA were evaluated. The importance of these evaluations lies on the fact that drugs used in treatments for cancer or with cytotoxic feature against tumor cell lines can cause problems to the functionality of important organs such as kidneys, liver and spleen (Saif, 2009). Hence, no significant change was observed in body mass variation. Also, no significant changes in the mass of livers, spleens and kidneys. In the peripheral blood from mice with sarcoma 180 tumor cells, the EOCA did not cause significant changes in total leukocyte counts after intraperitoneal administration.

6. Conclusions

The EOCA shows antioxidant activity and anticancer effects *in vitro* against 5 lines of tumor cells with greater potency to SF-295 and OVCAR-8. It is possible that these actions of the EOCA are related to the synergistic action of its constituents. However, no antitumor activity *in vivo* was seen against sarcoma 180 without showing toxic effects on parameters at the doses tested.

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