Physicochemical characteristics and cytotoxic effect of the methanolic extract of *Croton heliotrophiifolius* Kunth (Euphorbiaceae)

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Cytotoxic activity was evaluated in the following tumour cell lines; acute promyelocytic leukaemia (HL-60), human breast adenocarcinoma (MCF-7), human laryngeal carcinoma (Hep-2), human lung mucoepidermoid carcinoma (NCI-H292). Results revealed the presence of flavonoids and absence of alkaloids, coumarins, saponins and condensed tannins. HPLC identified Gallic acid with retention time of 1.80 min. The infrared spectrum identified the presence of esters group at 1661 cm⁻¹ of absorbance. On the other hand, the UV-Vis spectrum revealed bands of light absorbance on 268, 316, 406 nm indicating the presence of phenols and flavonoids. Results showed inhibition of cell growth of 59.5% on HL-60 cells, 46.5% on NCI-H292, 26.7% on Hep-2, and 21.7% on MCF-7 cells. In conclusion, the presence of phenolic compounds in the extract was demonstrated and a low to medium percentage of cell growth inhibition on tumour cell lines tested.

Key words: Croton, *Croton heliotrophiifolius*, flavonoids, gallic acid, cytotoxicity, tumour lines.

INTRODUCTION

The genre *Croton* comprises more than 1300 species of trees, bushes, and herbs distributes all over the world. Such biodiversity is mainly found in India, Brazil and Madagascar where its ethnomedicinal value is...
recognized (Yanpek et al., 2003; Ye et al., 2012). *Croton heliotropifolius* Kunth is an endemic species in the northeast of Brazil frequently found within “caatinga,” “brejo”, “resting” and “cerrado” vegetations. It is known by unofficial names such as “velame”, “velaminho” and “velame-de-cheiro” due to the presence of trichomes. Previous studies have described the presence of alkaloids, polyphenols and reducing agents in *C. heliotropifolius*, including its medicinal use for relief of stomach pain, vomit, diarrhea and as an anthithermic (Randau et al., 2004). The essential oil has been described as larvicidal against *Aedes aegypti* (Dória et al., 2010) and the ethanolic extract demonstrated significant insecticidal activity against *Sitophilus zeamais* (Silva et al., 2012). The Human Toxicity Potential (HTP) is an important test to be performed early in the study of a medicinal herb. It is recommended that new herbs and those with unknown actions shall be analysed in a controlled cellular environment as free of complex interactions inherent an organism. The methodologies is aimed at analysing the cellular behaviour, display several advantages such as low costs, easy and quick execution and controlled cell environment (Freshney, 2000). Cancer is a progressive chronic disease responsible for approximately 13% of deaths during last year (WHO, 2016). Genre, race, age, genetic predisposition and environmental exposure to carcinogenic agents, are directly related to the distribution and incidence of tumours (Chu et al., 2004).

Surgical removal of tumours combined with chemotherapy have been efficient methods in the treatment of several cancers. However, the appearance of side effects is very characteristic. Those effects very often compromise the continuity of the treatment which may lead to advanced stages of malignity and morbidity. Therefore, continuing efforts are focused towards the discovery of new antitumor compounds with more secure and efficient characteristics. In this context, the study of natural products seems promising (Erharuyi et al., 2017).

Due to knowledge that chemical analysis and the human toxicity potential are important assays for the medical use of a plant, this work aimed to characterize the physical-chemical properties of *C. heliotropifolius* leaves, and to evaluate its cytotoxic activity in several tumour cell lines.

**MATERIALS AND METHODS**

**General experimental procedures**

The methanolic extract of *C. heliotropifolius* leaves was concentrated using a rotary evaporator with vacuum BUCHI Switzerland at 60°C. Thin-layer chromatography (TLC) was performed with TLC Silicagel 60G F254 (Merck®) on top of an aluminium base. Compounds were sprayed with visualization reagents, visualized under UV light (254 nm - 365 nm), and for flavonoids, was compared with the quercetin standard. The HPLC system used was a HPLC Waters (Self Cleaning System) attached to an UV spectrophotometer with detector model Waters 2998 PDA containing photodiodes (Photodiode Array Detector). Values in the infrared region (FTIR) and UV-visible spectrum were registered respectively by an Agilent 630 and Shimadzu UV-vis 1800 spectrophotometers. All chemical products used in those procedures were for analytic use (Sigma® e Merck®). The Division of Antibiotics from the Federal University of Pernambuco (UFPE) supplied the cells, antibiotic solutions (penicillin and streptomycin), and the drug doxorubicin. Both culture media, Dulbecco MEM (DMEM) and Roswell Park Memorial Institute (RPMI), and the fetal bovine serum (FBS) were Gibco™ BRL. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was manufactured by Sigma®.

**Plant material**

*C. heliotropifolius* (Euphorbiaceae) leaves were collected in July 2015 in the urban area of Garanhuns city, Pernambuco State, Brazil (Latitude: -8.89074, Longitude: -36.4966 8° 53′ 27″ Sul, 36° 29′ 48″ Oeste). The plant was identified by the Dárdano de Andrade Lima herbarium in the Agronomic Research Institute (IPA). It was registered in the referred herbarium under the catalog number 90440 in which an exsiccate was deposited.

**Extraction of compounds**

The extract composed of plant leaves was prepared by soaking and maceration (Cechinel Filho and Yunes, 1998). About 150 g of powdered material was taken in a clean, flat bottomed glass container and soaked in 200 mL of methanol. The container with its contents was sealed and kept for a period of 10 days accompanying occasional shaking and stirring. Next, filtration was performed followed by evaporation of all solvent to obtain the crude extract. The presence of flavonoids, alkaloids and coumarins was tested by thin layer chromatography (TLC). The mobile phase used for flavonoids and alkaloids was ethyl acetate-formic acid-acetic acid-water (AcOEt- HCOOH-AcOH-H2O 100:11:12:77v/v), for coumarins ether-toluene- acetic acid 10% (50:50:50v/v) was used. The visualization reagents used for flavonoids and alkaloids were respectively ethylaminoester acid (Neu) and Dragendorff. For Coumarins, the visualization method used was UV light on 365 nm (Wagner et al., 1996). The presence of saponins was tested by mechanical shaking of the extract and visualization of foam (Simões et al., 2004). Formation of foam for 15 min was considered as positive for presence of saponins (Dewick, 2002). The presence of tannins was investigated by addition of iron chloride 0.5 M to the hydrated form of extract. In this method, the formation of blue precipitate indicates the presence of hydrolysed tannins awhile green precipitated the presence of condensed tannins (Matos, 1997). HPLC was conducted in model Shimadzu® HPLC LC-10, XBridge C18 column with 4.6 mm x 250 mm dimensions, 5 μm of particle size and 0.7 mL/min flow. The solvent system displayed: 94% of H2O and 6% of acetonitrile on 0 min (A), 65% of H2O and 35% of acetonitrile on 8 min (B), 94% of H2O and 6% of acetonitrile on 9 min (C); with 40 μL of injection volume and sample concentration of 20 mg/mL in 50% MeOH. Identification was performed using the standard method and external integration of the peaks at 256 nm for gallic acid. The spectroscopic profile of the extract was obtained by Fourier transform infrared spectroscopy (FTIR). In the semisol state, the extract was pressed against the diamond crystal of the equipment. Next, readings were recorded. The FTIR spectrum was obtained in less than 30 s and results compared against standards samples from the library. An aliquot (10 ml) of the extract (1 mg) diluted in MeOH (100%) was used to obtain the UV-visible spectrum, using the wavelength range of 200 to 700 nm.
Cytotoxic activity

Cytotoxic activity was tested by MTT assay on tumour cell lines. DMEM culture medium was used for incubation of NCI-H292 (human lung mucoepidermoid carcinoma) MCF-7 (human breast adenocarcinoma), and Hep-2 (human laryngeal carcinoma) cells. HL-60 (acute promyelocytic leukemia) cells were cultured in RPMI medium. In detail, all culture medium was supplemented with fetal bovine serum (10%) and antibiotic solution (1%) of penicillin and streptomycin. Cells were incubated on 37°C in rich humidity and 5% CO₂. Cells were cultured on a 96 well plate and incubated for 24 h prior to addition of 50 μg/ml of the extract dissolved in 1% DMSO. NCI-H292, HT-29, Hep-2 cell lines were incubated with initial concentration of 10⁵ cell/mL and HL-60 containing 3 x 10⁵ cell/mL. After a total of 72 h of incubation, 25 μL of MTT (5 mg/mL) was added to the culture. After 3 h of incubation, the culture medium was removed by aspiration, and 100 μl of DMSO (1%) added to each well. Control samples received doxorubicin (50 μg/mL) for inhibition purposes. This method enables the visualization of enzymatic activity by formation of purpura colour. A plate reader was used to obtain absorbance values in the wavelength of 560 nm and cell viability values expressed in percentage in comparison with a negative control, considered as “100% inhibited”. The experiment was performed with four sample replicates.

RESULTS AND DISCUSSION

Results from this study showed the methanolic extract of C. heliotropiifolius leaves have a chemical profile similar to its genre and to the majority of Euphorbiaceae family members. Thin-layer chromatography (TLC) identified the presence of flavonoids and the absence of alkaloids and coumarins. In addition, an alkaloid was identified in the root skin (Schoefs, 2002). The test for presence of saponins revealed their absence. In fact, such result has been reported in another specie of the genre, Croton linearifolius (Cunha et al., 2014). Experiments showed the extract did not contain condensed tannins although they have been detected in such specie (Randau et al., 2004).

High-performance liquid chromatography (HPLC) identified gallic acid with retention time of 1.80 min (Figure 1). The hydrolysis of esters bonds on gallic acid have been reported as a natural defence mechanism (Matias et al., 2010).

The spectrum within the infrared region (Figure 2) revealed a band in the region of 3271 cm⁻¹ suggesting the presence of O-H in association to amides. The band in the region of 2958 cm⁻¹ suggests the presence of C-H aliphatic. The region of 1661 cm⁻¹ shows the presence of carbonyl, aliphatic ketones and esters. Those compounds may be related to esters from the gallic acid. Absorbance
within the region of 1478 cm\textsuperscript{-1} refers to C=C. The range between 1140-1200 cm\textsuperscript{-1} indicates the presence of sulphones.

The UV spectrum (Figure 3) showed four absorbance bands with maximum values of 268, 316, 406 and 665 nm. Absorbance bands within 266-295 nm indicated the presence of simple phenols. The spectrum typically displays two maximum absorbance values for flavonoids; in 240-285 nm and 300-550 nm (Silva, 2015). Our results come in accordance to these values as demonstrated by


Table 1. Percentage of inhibitory activity of *C. heliotrophiifolius* extract on HL-60, MCF-7, Hep-2 e NCI-H292 cells.

| Cell line | *C. heliotrophiifolius* extract (%) | Doxorubicin (%) |
|-----------|------------------------------------|----------------|
| HL-60     | 59.5 (±2.9)                        | 92.9 (±0.6)    |
| MCF-7     | 21.7 (±3.7)                        | 79.4 (±2.6)    |
| Hep-2     | 26.7 (±7.1)                        | 79.4 (±2.6)    |
| NCI-H292  | 46.5 (±2.6)                        | 94.1 (±1.9)    |

chromatography and infrared spectrum results.

The toxicity of several secondary metabolites, present in vegetal, have been described (Silva et al., 2012). Flavonoids have their cytotoxic activity stablised in different tumour cells (Costa-Lotufo et al., 2003).

Experiments demonstrated cellular growth inhibition of 59.5, 21.7, 26.7 and 46.5% on the respective cell lines HL-60, MCF-7, Hep-2 e NCI-H292 (Table 1). Percentage values of 1-20% were classified as “without inhibitory activity”, between 20-50% as “low inhibitory activity”, for values between 50-70% as “moderate inhibitory activity”, and as “high inhibitory activity” for 70-100% (Andrade et al., 2015).

The extract here tested demonstrated to be more effective on acute promyelocytic leukaemia (HL-60) cells, with moderate growth inhibition. In a similar investigation, essential oil of *Croton zehntneri*, containing its major compound estragole, was tested on NCI-H292, MCF-7 and Hep-2 cell lines. Results revealed numbers lower than 20% of growth inhibition (Andrade et al., 2015). This study demonstrates that the physical-chemical profile of the extract is composed of phenolic compounds, flavonoids, and hydrolysed tannins. On the other hand, it avoid alkaloids and coumarins. The cytotoxic evaluation, within the conditions here tested, showed low inhibition rates on NCI-H292, MCF-7, Hep-2 cells but moderate on HL-60 cells which is potential for further investigations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations: TLC, Thin-layer Chromatography; HPLC, High-Performance Liquid Chromatography; FT-IR, Fourier Transformation Infrared spectroscopy; UV-Vis, Ultraviolet-visible spectrophotometry; MTT, bromo de 3-(4,5-dimetiltiazol-2-ii)-2,5-difeniltetrazólio; HL-60, acute promyelocytic leukaemia; MCF-7, human breast adenocarcinoma; hep-2, human laryngeal carcinoma; NCI-H292, human lung mucoepidermoid carcinoma.

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REFERENCES

Andrade TC, Lima SG, Freitas RM, Rocha MS, Islam T, Silva TG, Militao GC (2015). Isolation, characterization and evaluation of antimicrobial and cytotoxic activity of estragole, obtained from the essential oil of *croton zehntneri* (euphorbiaceae). An. Acad. Bras. Cienc. 87(1):173-182.

Cechinel Filho V, Yunes RA (1998). Strategies for obtaining compounds pharmacologically active from medicinal plants. Concepts about strategies to optimize the activity. Quim. Nova, 21(1):99-105.

Chu E, Sartorelli AC (2004). Cancer chemotherapy: In: Katzung BG, editor. Basic and clinical pharmacology, 9th ed. U.S.A. Portland, OR: McGraw-Hill. pp. 888-930.

Costa-Lotufo LV, Jimenez PC, Wille DV, Leal LKA, Cunha G, Silveira ER, Pessoa C (2003). Antiproliferative effects of several compounds isolated from *Amburana cearensis* AC Smith. Zeitschrift für Naturforschung C. 58(9-10):675-680.

Cunha SL, Gualberto SA, Carvalho KS, Fries DD (2014). Evaluation of the larvicidal activity of extracts obtained from Croton linearifolius Mull stem. Arg. (Euphorbiaceae) on larvae of *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae). Biotemas 27(2):79-85.

Dewick PM (2002). Medicinal natural products: a biosynthetic approach. John Wiley & Sons.

Dória GA, Silva WJ, Carvalho GA, Alves PB, Cavalcanti SC (2010). A study of the larvicidal activity of two *Croton* species from northeastern Brazil against *Aedes aegypti*. Pharm. Biol. 48(6):615-620.

Erharuyi O, Adhikari A, Falodun A, Jabeen A, Imam R, Ammad M, Gören N (2017). Cytotoxic, anti-inflammatory, and leishmanicidal activities of diterpenes isolated from the roots of *Caesalpinia pulcherrima*. Plant Med. 83(01/02):104-110.

Freshney RI (2000). Culture of animal cells: a manual of basic technique. 4a Edn. Indianapolis, In:WileyLiss.

Matias EF, katlucia Santos K, Almeida TS, Costa JG, Coutinho HD (2010). In vitro antibacterial activity of Croton campestris A., *Ocimum gratissimum* L. and Cordia venenacea DC. Rev. Bras. Biocienc. 8(3). Matos FJA (1997). Introduction to experimental phytochemical, 2ª edition., Ed. UFC: Fortaleza.

Randau KP, Florêncio DC, Ferreira CP, Xavier HS (2004). Pharmacognostic study of *Croton rhambinoloides* HBK and *Croton rhambinoloides* Pax & Hoffm.(Euphorbiaceae). Rev. Bras. Farmacogn. 14(2):89-96.

Schoefs B (2002). Chlorophyll and carotenoid analysis in food products. Properties of the pigments and methods of analysis. Trends Food Sci. Technol. 13(11):361-371.

Silva LB, Torres EB, Silva KF, Souza JSN, Lopes MS, Andrade LH, Xavier ZF (2012). Toxicity of ethanolic extract of *Croton heliotropifolius* in weevil populations of stored maize grains. J. Entomol. 6:413-421.

Silva RV (2015). Excerpt from the fruits of *Spondias purpurea* L. as the
active ingredient for formulation fitocosmética fotoprotetora. Feira de Santana. Dissertation, Estadual University of Feira de Santana.
Simões C, Schenkel EP, Gosmann G, Mello JCP, Mentz LA, Petrovick PR, (Orgs) (2004). Farmacognosia: da planta ao medicamento. 5 ed. rev. Porto Alegre/Florianópolis, Editora da UFRGS/Editora da UFSC.
Wagner H, Bladt S (1996). Plant drug analysis. 2.ed. New York: Springer Verlag.
World Health Organization (WHO) (1981). Intestinal protozoan and helminthic infections. Tech. Rep. Ser. 666:1-150.
Yankep E, Njamn D, Fotsing MT, Fomum ZT, Mbanya JC, Giner RM, Rios JL (2003). Griffonianone D, an Isoflavone with Anti-inflammatory Activity from the Root Bark of Millettia griffoniana 1. J. Nat. Prod. 66(9):1288-1290.
Ye H, Fu A, Wu W, Li Y, Wang G, Tang M, Yang J (2012). Cytotoxic and apoptotic effects of constituents from Millettia pachycarpa Benth. Fitoterapia 83(8):1402-1408.