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In vitro and in vivo anti-leukemia activity of the stem bark of *Salacia impressifolia* (Miers) A. C. Smith (Celastraceae)

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ABSTRACT

Ethnopharmacological relevance: *Salacia impressifolia* (Miers) A. C. Smith (family Celastraceae) is a traditional medicinal plant found in the Amazon Rainforest known as “miraruíra”, "cipó-miraruíra" or “panu” and is traditionally used to treat dengue, flu, inflammation, pain, diabetes, male impotency, renal affections, rheumatism and cancer. *Aim of the study:* The aim of this study was to investigate in vitro and in vivo anti-leukemia activity of the stem bark of *S. impressifolia* in experimental models. *Materials and methods:* The in vitro cytotoxic activity of extracts, fractions and quinonemethide triterpenes (22-hydroxytingenone, tingenone and pristimerin) from the stem bark of *S. impressifolia* in cultured cancer cells was determined. The in vivo antitumor activity of the ethyl acetate extract (EAE) and of its fraction (FEAE.3) from the stem bark of *S. impressifolia* was assessed in C.B-17 severe combined immunodeficient (SCID) mice engrafted with human promyelocytic leukemia HL-60 cells. *Results:* The extract EAE, its fraction FEAE.3, and quinonemethide triterpenes exhibited potent cytotoxicity against cancer cell lines, including in vitro anti-leukemia activity against HL-60 and K-562 cells. Moreover, extract EAE and its fraction FEAE.3 inhibited the in vivo development of HL-60 cells engrafted in C.B-17 SCID mice. Tumor mass inhibition rates were measured as 40.4% and 81.5% for the extract EAE (20 mg/kg) and for its fraction FEAE.3 (20 mg/kg), respectively. *Conclusions:* Ethyl acetate extract and its fraction from the stem bark of *S. impressifolia* exhibit in vitro and in vivo anti-leukemia activity that can be attributed to their quinonemethide triterpenes. These data confirm the ethnopharmacological use of this species and may contribute to the development of a novel anticancer herbal medicine.

1. Introduction

The genus *Salacia* (family Celastraceae) includes approximately 200 species distributed throughout the Americas, Asia and Europe (Udayan et al., 2012). The genus is of great ethnopharmacological relevance, as numerous plants belonging to it exhibit pharmacological properties including anti-diabetic (Stohs and Ray, 2015; Shirakawa et al., 2016), anti-hyperlipidemic (Stohs and Ray, 2015), immunomodulation (Oda et al., 2015), antiviral (Romero-Pérez et al., 2016; Ferreira et al., 2018), antioxidant (Ghadage et al., 2017), antimutagenicity (Carneiro et al., 2018) effects. In particular, anticancer potential has been reported for *S. oblonga* (Musini et al., 2015), *S. leptoclada* (Ruphin et al., 2013), *S.*

Abbreviations: 5-FU, 5-fluorouracil; ANOVA, analysis of variance; ATCC, American Type Culture Collection; CC, column chromatography; CTL, negative control; DMSO, dimethyl sulfoxide; DOX, doxorubicin; EAE, ethyl acetate extract; FEAE.3, fraction of the ethyl acetate extract; FME.1, fraction of the methanol extract; HE, hexane extract; HPLC, high-performance liquid chromatography; IC_{50}, half maximal inhibitory concentration; INPA, National Institute of Research in the Amazon; IR, infrared; ME, methanol extract; NMR, nuclear magnetic resonance; PBMC, peripheral blood mononuclear cells; SCID, severe combined immunodeficient; UV, ultraviolet

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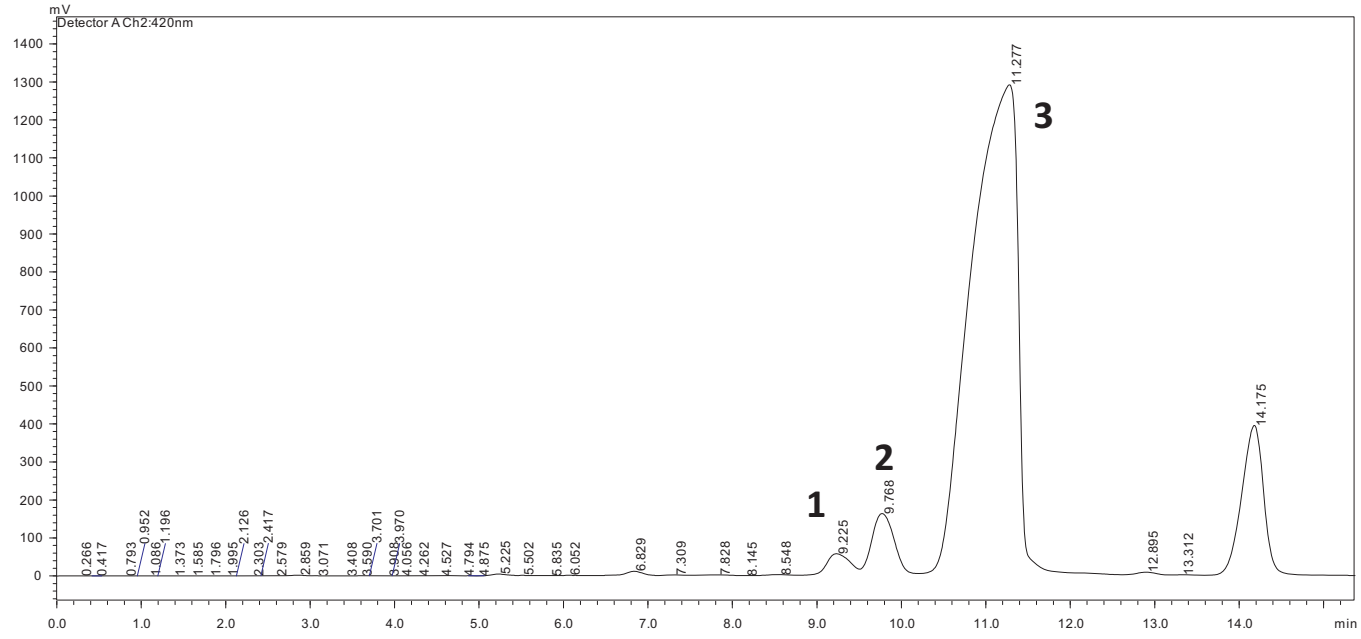

Fig. 1. Reverse phase semi-preparative chromatogram of fraction FEAE.3 at 420 nm.

verrucosa (Somwong et al., 2011), S. *madagascariensis* (Sneden, 1981) and *S.* sp. near *petenensis* (Setzer et al., 1998).

The species *Salacia impressifolia* (Miers) A. C. Smith is distributed throughout Central and South America and can be found in the Brazilian states of Acre, Amazonas, Pará, Rondônia, Roraima, and Mato Grosso (Lombardi, 2010). In Brazil, *S. impressifolia* is popularly known as “miraruíra” or "cipó-miraruíra," and its stalks are used to treat inflammation and diabetes (Almeida, 1993; Lorenzi and Matos, 2002). In the Amazonian ethnic group Tacana from Bolivia, *S. impressifolia* is known as “panu,” and its bark is prepared by decoction to treat general body pain or is macerated in alcohol for use as general restorative medicine or to treat male impotency and rheumatic pain (Bourdy et al., 2000). Moreover, a syrup is prepared to treat kidney pain and the flu (Bourdy et al., 2000). In the Peruvian Amazon it is also known as “panu” and is used to treat dengue, renal affections, rheumatism and cancer (Brako and Zarucchi, 1993; Clavo et al., 2003).

Although several medicinal uses of *S. impressifolia* have been discovered, few scientific works have focused on this species, for which the antioxidant properties and some of its chemical constituents have been reported. The infusion extract drawn from stems of *S. impressifolia* has antioxidant effects (Santo et al., 2013; Manoel and Moya, 2015), and 15 compounds have been isolated from the trunk and twigs of *S. impressifolia*, among which pristimerin and tingenone have been isolated as major constituents along with friedelin, α-amyrin, β-amyrin, β-sitosterol, lupeol, 2-oxo-20(29)-lupen-3β-ol, salicinin B, 2β,3β-dihydroxylup-20(29)-ene, 30-hydroxypristimerin, isoiguesterin, 22-hydroxytingenone, regeol A, and netzahualcoyene (Silva et al., 2016). In addition, from *S. impressifolia* roots, friedelin, β-sitosterol, quinovic acid, cincholic acid, cincholic-3β-O-6-deoxy-β-D-glucopyranoside acid, quinovic-3β-O-β-D-glucopyranoside acid and celastrol have been isolated (Paz et al., 2018). The aim of this study was to investigate the in vitro and in vivo anti-leukemia activity of stem bark of *S. impressifolia* using experimental models.

2. Material and methods

2.1. Plant material

Stems of a flowering specimen of *S. impressifolia* were collected in April 2014 from the jungle of the Adolpho Ducke Botanical Garden (coordinates 2°56′58.2″ S, 59°56′36.3″ W) in the municipality of Manaus, Amazonas State, Brazil. The plant material was identified at the herbarium of the National Institute of Research in the Amazon (INPA) (voucher code #4699).

2.2. Preparation of extracts and compound purification

Air-dried and powdered plant material of *S. impressifolia* (900 g) was sequentially macerated at room temperature (c.a. 26 °C) with 2 L of *n*-hexane, ethyl acetate, and methanol (3 x to each solvent). The resulting extracts were evaporated at lower pressure until dry to form *n*-hexane extract (HE, 12.5 g, 1,38%), ethyl acetate extract (EAE, 87.8 g, 9.75%), and methanol extract (ME, 102.2 g, 11.3%). Some EAE (15 g) was fractionated via silica gel column chromatography (CC) with increasing concentrations of dichloromethane-ethyl acetate (100:0, 50:50, and 0:100) and ethyl acetate-methanol (100:0, 50:50, and 0:100), forming six fractions of FEAE.1–6. A portion of extract ME (10 g) was subjected to silica gel CC with gradient systems of ethyl acetate-acetone (100:0, 50:50, and 0:100) and acetone-methanol (100:0, 50:50, and 0:100), forming six main fractions of FME.1–6. Some of the active fraction FEAE.3 (250 mg, 10 injections of 25 mg) was subjected tosemi-preparative high-performance liquid chromatography (HPLC) purification using a Shimadzu ultra-fast liquid chromatograph (UFLC) system (Kyoto, Japan) with an isocratic elution consisting of acetonitrile/water 85:15 (v:v) fitted over a Shimadzu Shim-pack C18 reversed-phase column (10 mm × 250 mm, 5 μm) at a flow rate of 8.0 mL/min and UV detection at 420 nm. We in turn isolated 22-hydroxytingenone (**1**, t_R 9.22 min, 6.40 mg), tingenone (**2**, t_R 9.77 min, 11.0 mg), and pristimerin (**3**, 11.28 min, 53.5 mg) (Silva et al., 2016) (Fig. 1). The chemical structures of 22-hydroxytingenone, tingenone and pristimerin are shown in Fig. 2. Ultraviolet (UV), infrared (IR) and nuclear magnetic resonance (NMR) spectra for pure compounds are available in the supplementary materials section (Figs. S1–13).

2.3. In vitro assays

2.3.1. Cells

Cancer cells MCF-7 (human breast adenocarcinoma), HCT116 (human colon carcinoma), HepG2 (human hepatocellular carcinoma), SCC-4 (human oral squamous cell carcinoma), HSC-3 (human oral

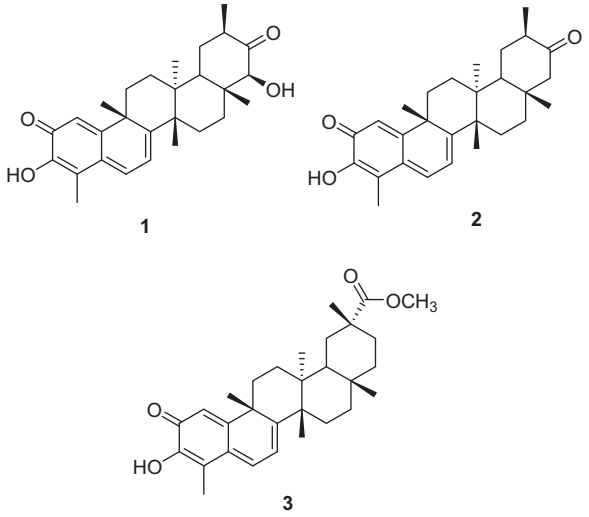

Fig. 2. Chemical structures of 22-hydroxytingenone (**1**), tingenone (**2**) and pristimerin (**3**).

squamous cell carcinoma), HL-60 (human promyelocytic leukemia), K-562 (human chronic myelogenous leukemia) and B16-F10 (mouse melanoma) and noncancerous cell MRC-5 (human lung fibroblast) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured as recommended by the ATCC. Primary human peripheral blood mononuclear cells (PBMC) were obtained using a standard ficoll density protocol, and the Research Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved of the experimental protocol employed (#031019/2013). Cell viability was examined via trypan blue exclusion assay for all experiments.

2.3.2. Cytotoxicity assay

Cell viability was quantified using an Alamar blue assay and was performed following a procedure previously described (Ahmed et al., 1994). In brief, cells were inserted into 96-well plates for all experiments (7×10^4 cells/mL for adherent cells or 3×10^5 cells/mL for suspended cells in 100 µL of medium). After 24 h the samples were dissolved in dimethyl sulfoxide (DMSO) and added to each well and incubated for 72 h. Doxorubicin (purity ≥ 95%, doxorubicin hydrochloride, Laboratory IMA S.A.I.C., Buenos Aires, Argentina) was used as the positive control. Four (for cell lines) or 24 h (for PBMCs) before the end of incubation, 20 µL of a stock solution (0.312 mg/mL) of Alamar blue (resazurin, Sigma-Aldrich Co., Saint Louis, MO, USA) was added to each well. Absorbance at 570 nm and 600 nm was measured using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, EUA), and the drug effect was quantified as the percentage of control absorbance.

2.4. In vivo assays

2.4.1. Animals

In total, 70 C.B-17 severe combined immunodeficient (SCID) mice (females, 25–30 g) were obtained and maintained at Gonçalo Moniz Institute-FIOCRUZ animal facilities (Salvador, Bahia, Brazil). Animals were housed in cages with free access to food and water. All animals were subjected to a 12:12 h light-dark cycle (lights on at 6:00 a.m.). A local animal ethics committee approved of the experimental protocol employed (number #06/2015).

2.4.2. Human leukemia xenograft model

HL-60 cells (1.5×10^7 cells per 500 µL) were implanted subcutaneously into the left front armpits of the mice. At the beginning of the experiment, mice were randomly divided into five groups: group 1 animals received injections of vehicle with 5% DMSO solution ($n = 22$); group 2 animals received injections of doxorubicin (0.8 mg/kg, $n = 10$); group 3 animals received injections of 5-fluorouracil (15 mg/kg, Sigma-Aldrich, $n = 11$); group 4 animals received injections of EAE at 20 mg/kg ($n = 12$); and group 5 animals received injections of FEAE.3 at 20 mg/kg ($n = 10$). These doses were selected based in previous works using herbal extracts in vivo tumor models (Mousinho et al., 2011; Dória et al., 2016; Ferreira et al., 2016). When the tumors reached 100–200 mm^3, the animals were treated through the intraperitoneal route (200 µL per animal) once daily for 15 consecutive days. One day after the end of the treatment, the animals were anesthetized, and peripheral blood samples were collected from the brachial artery. Animals were euthanized by anesthetic overdose, and tumors were excised and weighed.

2.4.3. Toxicological evaluation

To assess toxicological effects, mice were weighed at the beginning and end of the experiment. Animals were observed for signs of abnormality throughout the study. A hematological analysis was performed using the Advia 60 hematology system (Bayer, Leverkusen, Germany). Livers, kidneys, lungs and hearts were removed, weighed and examined for signs of gross lesion formation, color change and/or hemorrhaging. After gross macroscopic examination, the tumors, livers, kidneys, lungs and hearts were fixed in 4% formaldehyde and embedded in paraffin. Tissue sections were stained with hematoxylin–eosin and Periodic acid-Schiff (liver and kidneys), and histological analyses were performed under optical microscopy by an experienced pathologist.

2.5. Statistical analysis

Data are presented as means ± S.E.M. or as half maximal inhibitory concentrations (IC_{50}) with 95% confidence intervals obtained by non-linear regression. Differences between the experimental groups were compared through an analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test ($P < 0.05$). All statistical analyses were performed using GraphPad Prism (Intuitive Software for Science; San Diego, CA, USA).

3. Results

3.1. In vitro cytotoxicity

The in vitro cytotoxic activity of extracts EAE and ME and of their respective fractions FEAE.3 and FME.1 and quinonemethide triterpenes (22-hydroxytingenone, tingenone and pristimerin) from the stem bark of *S. impressifolia* was determined in cultured cancer cells (MCF-7, HCT116, HepG2, SCC-4, HSC-3, HL-60, K-562 and B16-F10) after 72 h of incubation using the alamar blue assay. Fig. 3 presents the cell survival curves obtained. Extract EAE, its fraction FEAE.3, and quinonemethide triterpenes 22-hydroxytingenone and tingenone showed the most potent levels of cytotoxicity.

IC_{50} values of the cytotoxic activity of extracts EAE and ME and of respective fractions FEAE.3 and FME.1 are shown in Table 1. Extract EAE presented the lowest IC_{50} value in the cancer cell MCF-7 (8.4 µg/mL) and the highest IC_{50} value in the cancer cell K-562 (29.5 µg/mL) while extract ME showed IC_{50} values of > 50 µg/mL for all cancer cell lines tested. Fraction FEAE.3 presented the lowest IC_{50} value in cancer cell HepG2 (0.1 µg/mL) and the highest IC_{50} value in cancer cell HSC-3 (0.8 µg/mL), and fraction FME.1 presented the lowest IC_{50} value in cancer cell HepG2/B16-F10 (0.5 µg/mL) and the highest IC_{50} value in cancer cell K-562 (1.0 µg/mL).

IC_{50} values reflecting the cytotoxic activity of quinonemethide triterpenes 22-hydroxytingenone, tingenone and pristimerin were also obtained and are shown in Table 2. 22-Hydroxytingenone presented the

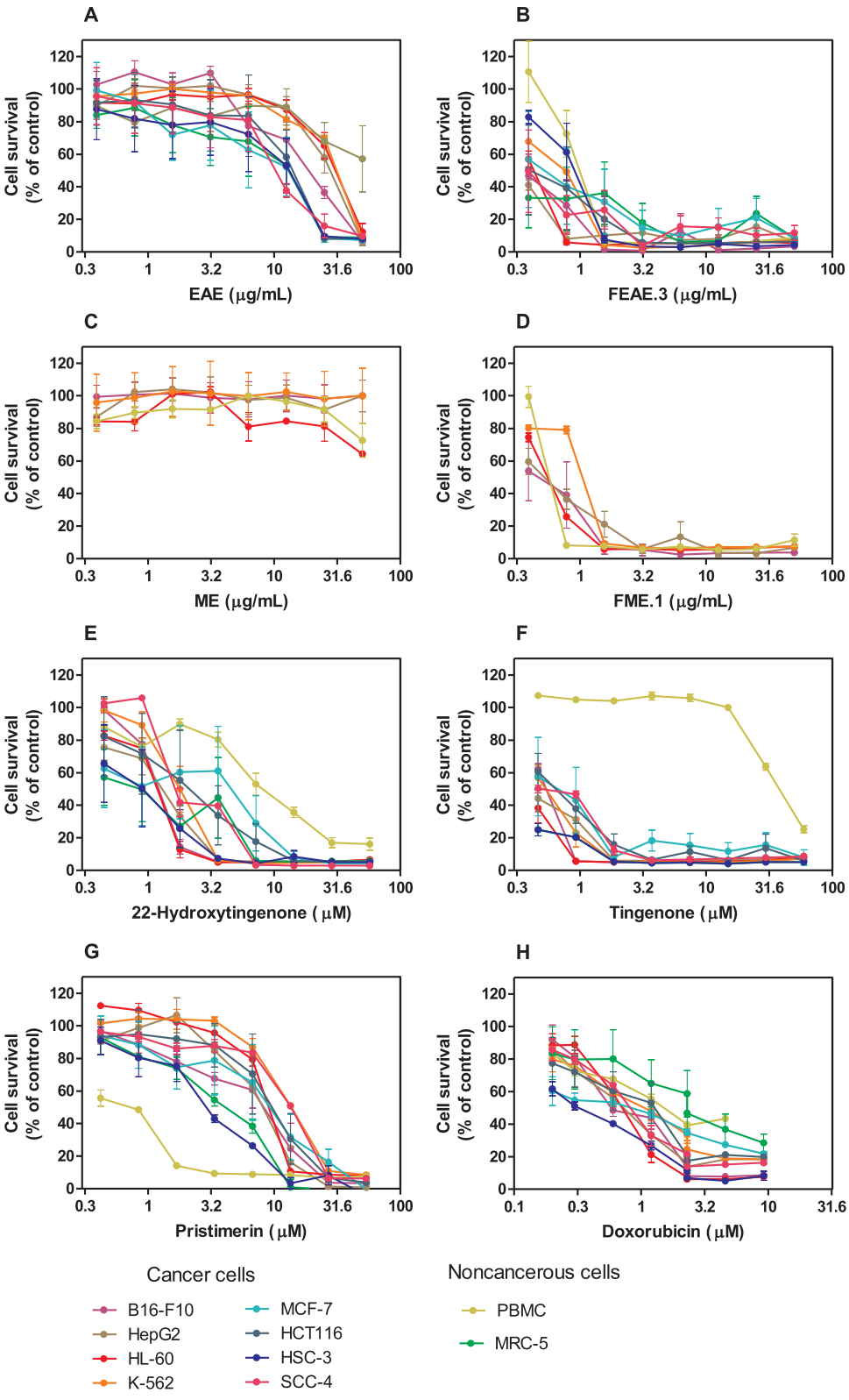

Fig. 3. Cell survival curves obtained from three independent experiments performed in duplicate and measured by alamar blue assay after 72 h of incubation. Cancer cells: MCF-7 (human breast adenocarcinoma); HCT116 (human colon carcinoma); HepG2 (human hepatocellular carcinoma); SCC-4 (human oral squamous cell carcinoma); HSC-3 (human oral squamous cell carcinoma); HL-60 (human promyelocytic leukemia); K-562 (human chronic myelogenous leukemia); and B16-F10 (mouse melanoma). Noncancerous cells: MRC-5 (human lung fibroblast) and PBMC (human peripheral blood mononuclear cells). EAE: Ethyl acetate extract. ME: Methanol extract. FEAE.3: Fraction of the ethyl acetate extract. FME.1: Fraction of the methanol extract.

lowest IC_{50} value in cancer cell HSC-3 (0.8 μM) and the highest IC_{50} value in cancer cell SCC-4 (2.0 μM). Tingenone presented the lowest IC_{50} value in cancer cell HSC-3 (0.1 μM) and the highest IC_{50} value in cancer cell HCT116/SCC-4 (0.6 μM). Finally, pristimerin presented the lowest IC_{50} value in cancer cell HSC-3 (2.9 μM) and the highest IC_{50} value in cancer cell SCC-4 (12.7 μM). Doxorubicin was used as the positive control and showed the lowest IC_{50} value in cancer cell HSC-3 (0.3 μM) and the highest IC_{50} value in cancer cell K-562 (1.0 μM).

In addition to applying a cytotoxicity assay of cancer cells, the cytotoxic effect of extracts EAE and ME, of their respective fractions FEAE.3 and FME.1 and of quinonemethide triterpenes was also evaluated in noncancerous cells (MRC-5 and PBMC), and were cytotoxic to these cells, indicating low selectively for cancer cells. Doxorubicin, a clinically useful chemotherapy drug, also exhibited cytotoxicity to

Table 1
IC_{50} values (in μg/mL) of the cytotoxic activity of the extracts and fractions from the stem bark of *Salacia impressifolia*.

Cells	IC_{50} (95% CI)			
	EAE	ME	FEAE.3	FME.1
Cancer cells				
MCF-7	8.4 (4.6–15.5)	N.d.	0.4 (0.1 – 2.0)	N.d.
HCT116	12.7 (8.3–19.5)	N.d.	0.4 (0.2 – 1.0)	N.d.
HepG2	26.6 (23.1–30.6)	> 50	0.1 (0.1–0.5)	0.5 (0.4–0.7)
SCC-4	10.4 (6.7–16.1)	N.d.	0.2 (0.1–1.2)	N.d.
HSC-3	9.8 (5.4–17.8)	N.d.	0.8 (0.7 – 1.0)	N.d.
HL-60	29.1 (25.4–33.4)	> 50	0.4 (0.4–0.5)	0.6 (0.5–0.6)
K-562	29.5 (26.0–33.4)	> 50	0.6 (0.6–0.8)	1.0 (0.9–1.2)
B16-F10	17.7 (14.4–21.7)	> 50	0.4 (0.3–0.6)	0.5 (0.3–0.7)
Noncancerous cells				
MRC-5	8.4 (4.6–15.2)	N.d.	0.1 (0.1–0.3)	N.d.
PBMC	> 50	> 50	0.9 (0.8–1.1)	0.6 (0.4–1.0)

Data are presented as IC_{50} values, in μg/mL, with respective 95% confidence interval (95% CI) obtained by nonlinear regression from at the least three independent experiments performed in duplicate, measured by alamar blue assay after 72 h of incubation. Cancer cells: MCF-7 (human breast adenocarcinoma); HCT116 (human colon carcinoma); HepG2 (human hepatocellular carcinoma); SCC-4 (human oral squamous cell carcinoma); HSC-3 (human oral squamous cell carcinoma); HL-60 (human promyelocytic leukemia); K-562 (human chronic myelogenous leukemia); and B16-F10 (mouse melanoma). Noncancerous cells: MRC-5 (human lung fibroblast) and PBMC (human peripheral blood mononuclear cells). EAE: Ethyl acetate extract. ME: Methanol extract. FEAE.3: Fraction of the ethyl acetate extract. FME.1: Fraction of the methanol extract. N.d. Not determined.

noncancer cells.

The human leukemia HL-60 cell line is a cellular model often used to study of the antileukemia activity of new compounds (Magalhães et al., 2013; Yun et al., 2017; Calgarotto et al., 2018; Oliveira et al., 2018). Moreover, this cell line is sensitive to extract EAE and to its fraction FEAE.3. Therefore, the cell line HL-60 was used in an in vivo study.

3.2. In vivo anti-leukemia activity

The in vivo anti-leukemia activity of extract EAE and of its fraction FEAE.3 drawn from the stem bark of *S. impressifolia* was investigated in C.B-17 SCID mice engrafted with HL-60 cells. The animals were treated with extract EAE (20 mg/kg) or with its fraction FEAE.3 (20 mg/kg) through intraperitoneal injections delivered once a day for 15 consecutive days. By the end of the treatment, the mean tumor mass weight of the negative control animals was 5.23 ± 0.49 g (Fig. 4A). In EAE-treated animals, the mean tumor mass weights was 3.12 ± 0.320 while it was measured as 0.97 ± 0.41 g for animals treated with fraction FEAE.3. Tumor mass inhibition was measured as 40.4% and 81.5% for extract EAE and its fraction FEAE.3, respectively (Fig. 4B). The positive controls (0.8 mg/kg doxorubicin and 15 mg/kg 5-fluorouracil) reduced the tumor weight by 70.7% and 53.9%, respectively.

A histology analysis of the tumors was also performed (Fig. 4C). We observed loose clusters of atypical cells of myeloid lineage exhibiting blast appearance, loose chromatin, clear nucleoli, basophilic cytoplasm, and varying degrees of cytoplasmic granulation in all experimental groups. Although necrosis and apoptosis were observed in all of the groups, we observed more karyorrhexis and apoptosis in the doxorubicin and FEAE.3 groups. In addition, the occurrence of bleeding was an important finding for the EAE and FEAE.3 groups.

Toxicological factors were also assessed in C.B-17 SCID mice with HL-60 cell xenografts. A decrease in body weight was observed in the animals treated with EAE, FEAE.3 and doxorubicin ($P < 0.05$). No significant alterations were observed in the liver, kidney, lung or heart wet weights of any group ($P > 0.05$) (Table 3). Hematological parameters of peripheral blood drawn from C.B-17 SCID mice with HL-60 cell xenografts were analyzed (Table 4). All hematological parameters remained unchanged after treatment with EAE and FEAE.3 ($P > 0.05$). On the other hand, a decline in the number of platelets and hematocrit in the doxorubicin group was observed when compared to those of the negative control group ($P < 0.05$).

Morphological analyses of liver, kidney, lung, and heart tissue for each group were also conducted (Fig. S14). In liver tissue, the acinar architecture and centrilobular vein were preserved in all groups. Inflammation observed in the liver portal space was discrete in most animals. Other symptoms such as congestion and hydropic degeneration were found in all groups at mild to moderate levels. In addition, focal areas of coagulation necrosis were observed in all groups and metastatic nodules were detected in all animals of group FEAE.3.

In the kidneys of animals, histopathological changes involved vascular congestion and the thickening of the basal membrane of the renal glomerulus with decreased urinary space, which were observed in all groups treated with the compounds. Furthermore, discrete coagulation necrosis was observed in the medullary region of the kidneys of certain animals treated with doxorubicin and 5-fluorouracil. However, tissue architectures were maintained in all experimental groups.

In the lungs, architectures of the parenchyma ranged from partially

Table 2
IC_{50} values (in μM) of the cytotoxic activity of the quinonemethide triterpenes from the stem bark of *Salacia impressifolia*.

Cells	IC_{50} (95% CI)			
	22-Hydroxytingenone	Tingenone	Pristimerin	Doxorubicin
Cancer cells				
MCF-7	1.9 (0.7–5.3)	0.5 (0.2–1.4)	7.9 (4.7–13.4)	0.6 (0.5–0.8)
HCT116	2.0 (1.0–3.8)	0.6 (0.5–0.9)	9.4 (6.6–13.3)	0.9 (0.6–1.5)
HepG2	1.2 (1.0–1.4)	0.4 (0.3–0.6)	7.8 (6.8–8.9)	0,7 (0.6–0.9)
SCC-4	2.2 (1.7–2.8)	0.6 (0.4–0.8)	12.7 (10.9–14.8)	0.8 (0.7 – 1.0)
HSC-3	0.8 (0.5–1.3)	0.1 (0.1–0.3)	2.9 (2.3–3.7)	0.3 (0.3–0.4)
HL-60	1.1 (1.1–1.3)	0.4 (0.3–0.5)	8.8 (7.8–10.1)	0.7 (0.6–0.8)
K-562	1.8 (1.5–2.0)	0.5 (0.5–0.6)	13.6 (12.3–15.0)	1.0 (0.8–1.2)
B16-F10	1.2 (1.1–1.3)	0.5 (0.5–0.5)	6.3 (4.7–8.4)	0.7 (0.6–0.9)
Noncancerous cells				
MRC-5	0,8 (0.3–1.9)	0.1 (0.1–0.2)	3.5 (2.9–4.2)	2.6 (1.4–4.9)
PBMC	8.6 (6.5–11.2)	38.5 (34.1–43.6)	0.6 (0.4–0.7)	1.7 (1.3–2.1)

Data are presented as IC_{50} values, in μM, with respective 95% confidence interval (95% CI) obtained by nonlinear regression from at the least three independent experiments performed in duplicate, measured by alamar blue assay after 72 h of incubation. Cancer cells: MCF-7 (human breast adenocarcinoma); HCT116 (human colon carcinoma); HepG2 (human hepatocellular carcinoma); SCC-4 (human oral squamous cell carcinoma); HSC-3 (human oral squamous cell carcinoma); HL-60 (human promyelocytic leukemia); K-562 (human chronic myelogenous leukemia); and B16-F10 (mouse melanoma). Noncancerous cells: MRC-5 (human lung fibroblast); and PBMC (human peripheral blood mononuclear cells). Doxorubicin was used as the positive control.

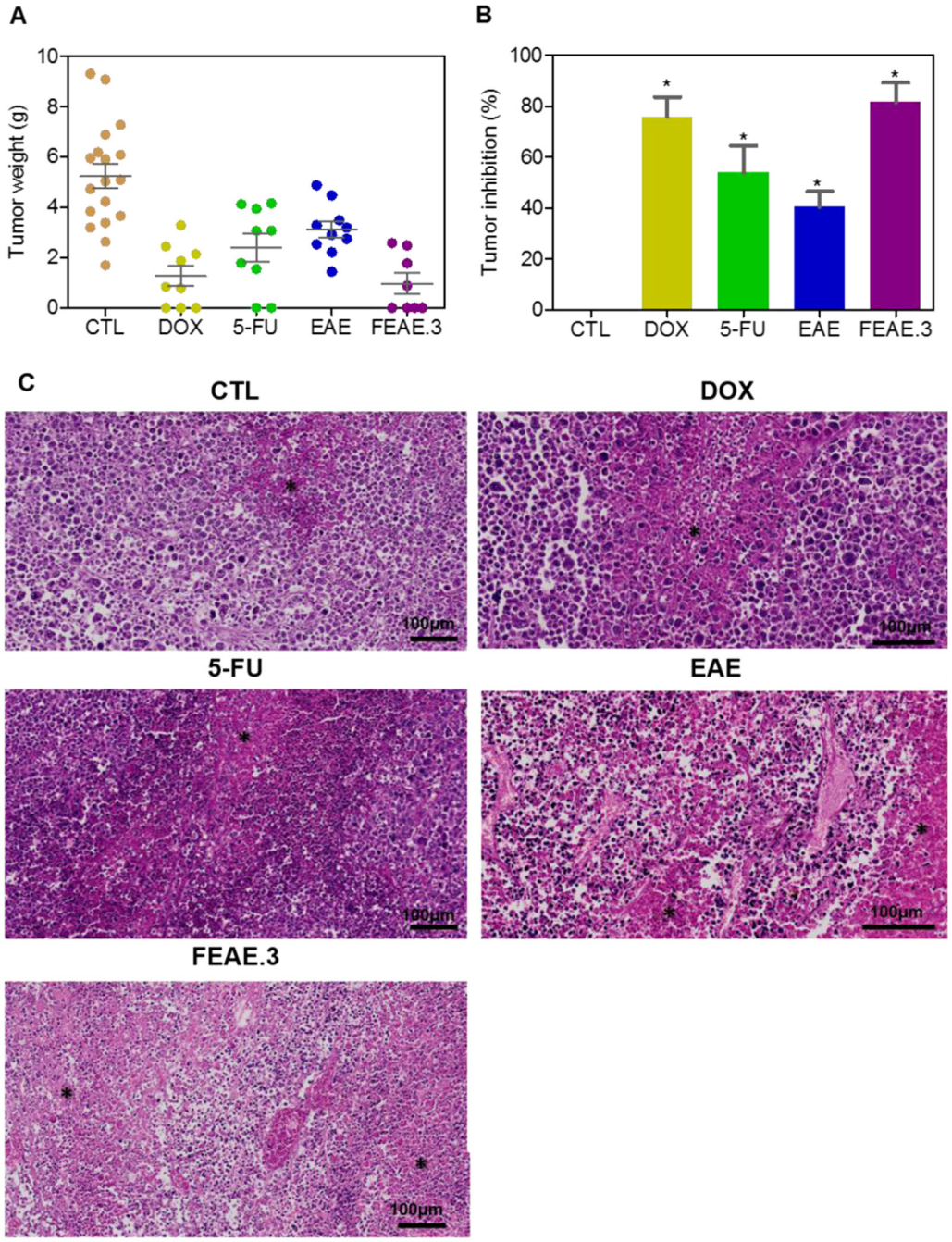

Fig. 4. In vivo antitumor activity of the ethyl acetate extract (EAE) and of its fraction (FEAE.3) from the stem bark of *Salacia impressifolia* in C.B-17 SCID mice with HL-60 cell xenografts. (A) Tumor weight (g) after treatment. (B) Tumor inhibition (%) after treatment. Data are presented as means ± S.E.M. of 8–21 animals. *$P < 0.05$ compared to the negative control by ANOVA followed by Bonferroni's Multiple Comparison Test. (C) Representative histological analysis of tumors stained with hematoxylin and eosin and analyzed by light microscopy (bar = 100 µm). The asterisks represent areas with tumor necrosis. When the tumors reached 100–200 mm^3, the animals were treated through the intraperitoneal route for 15 consecutive days with EAE (20 mg/kg) and FEAE.3 (20 mg/kg). The negative control (CTL) was treated with the vehicle (5% DMSO) used to dilute the tested substances. Doxorubicin (DOX, 0.8 mg/kg) and 5-fluorouracil (5-FU, 15 mg/kg) were used as positive controls.

Table 3
Effect of the ethyl acetate extract (EAE) and of its fraction (FEAE.3) from the stem bark of *Salacia impressifolia* on body and relative organ weight from C.B-17 SCID mice with HL-60 cell xenografts.

Parameters	CTL	DOX	5-FU	EAE	FEAE.3
Dose (mg/kg)	–	0.8	15	20	20
Survival	21/22	10/10	11/11	12/12	8/10
Initial body weight (g)	24.5 ± 0.2	24.5 ± 0.3	25.4 ± 0.6	24.0 ± 0.5	23.5 ± 0.6
Final body weight (g)	25.6 ± 0.4	20.3 ± 0.7*	25.6 ± 0.4	22.5 ± 0.8*	17.6 ± 0.5*
Liver (g/100 g body weight)	5.5 ± 0.3	5.5 ± 0.4	5.3 ± 0.3	5.7 ± 0.4	4.6 ± 0.4
Kidney (g/100 g body weight)	1.4 ± 0.04	1.6 ± 0.1	1.4 ± 0.04	1.4 ± 0.1	1.6 ± 0.1
Heart (g/100 g body weight)	0.6 ± 0.2	0.5 ± 0.04	0.5 ± 0.02	0.5 ± 0.04	0.7 ± 0.1
Lung (g/100 g body weight)	0.7 ± 0.02	0.8 ± 0.03	0.7 ± 0.04	0.8 ± 0.1	0.8 ± 0.1

When the tumors reached 100–200 mm^3, the animals were treated through the intraperitoneal route for 15 consecutive days. The negative control (CTL) was treated with the vehicle (5% DMSO) used to dilute the tested substances. Doxorubicin (DOX) and 5-fluorouracil (5-FU) were used as positive controls. Data are presented as means ± S.E.M. of 8–21 animals.

* $P < 0.05$ compared to the negative control by ANOVA followed by Bonferroni's Multiple Comparison Test.

Table 4
Effect of the ethyl acetate extract (EAE) and of its fraction (FEAE.3) from the stem bark of *Salacia impressifolia* on hematological parameters of peripheral blood from C.B-17 SCID mice with HL-60 cell xenografts.

Parameters	Non-tumor	CTL	DOX	5-FU	EAE	FEAE.3
Dose (mg/kg)	–	–	0.8	15	20	20
Erythrocytes (10^6/mm^3)	10.8 ± 0.2	10.0 ± 0.3	10.1 ± 0.5	9.4 ± 0.3	10.3 ± 0.3	10.8 ± 0.6
Hemoglobin (g/dL)	13.7 ± 0.2	13.9 ± 0.5	12.2 ± 0.3	12.1 ± 0.8	12.8 ± 0.3	14.2 ± 0.7
Hematocrit (%)	56.0 ± 1.0	52.0 ± 1.1	46.0 ± 1.5*	47.3 ± 0.6	50.1 ± 1.3	48.8 ± 3.4
MCV (fL)	54.0 ± 0.3	47.8 ± 0.3	46.4 ± 0.4	47.9 ± 0.3	49.1 ± 0.1	47.6 ± 0.5
Platelets (10^3/mm^3)	750.8 ± 41.0	759.6 ± 111.9	343.0 ± 65.9*	479.9 ± 47.9	654.0 ± 39.0	506.1 ± 50.6
Leukocytes (10^3/mm^3)	1.4 ± 0.3	3.4 ± 0.6	1.5 ± 0.2	1.3 ± 0.2	4.8 ± 1.4	5.4 ± 1.2
Differential leukocytes (%)						
Granulocytes	17.2 ± 4.5	36.3 ± 3.1	37.6 ± 3.5	36.0 ± 4.1	45.9 ± 8.7	50.1 ± 1.8
Lymphocytes	64.6 ± 4.7	24.0 ± 3.5	36.0 ± 6.6	39.3 ± 7.0	27.8 ± 10.9	8.0 ± 0.7
Monocytes	18.3 ± 0.2	32.9 ± 1.8	26.5 ± 5.1	24.5 ± 2.9	26.1 ± 3.2	41.8 ± 1.7

Non-tumor group represents C.B-17 SCID mice without tumor inoculation or any treatment. When the tumors reached 100–200 mm^3, the animals were treated through the intraperitoneal route for 15 consecutive days. The negative control (CTL) was treated with the vehicle (5% DMSO) used to dilute the tested substances. Doxorubicin (DOX) and 5-fluorouracil (5-FU) was used as positive controls. Data are presented as means ± S.E.M. of 5–13 animals.

* $P < 0.05$ compared with to negative control by ANOVA, followed by Bonferroni's Multiple Comparison Test. MCV: Mean Corpuscular Volume.

maintained to modified in all groups. Histopathological changes observed in all animals ranged from mild to severe. Significant acute inflammation, edema, congestion, hemorrhaging, and the thickening of the alveolar septum with decreased airspace were frequently observed. A histopathological analysis of animal hearts did not reveal alterations in any group.

Interestingly, most of these histopathological alterations, including those involving hydropic degeneration, vascular congestion and focal areas of inflammation, are acute cellular responses. Therefore, when treatment is concluded, the organism can return to a state of homeostasis, as most of these disturbances are reversible.

4. Discussion

Medicinal plants have traditionally been used in the realms of anticancer drug discovery and development. The discovery and development of standardized extracts or fractions and of single pure compounds isolated from medicinal plants with cytotoxicity are central to cancer treatment. The present work is the first to describe the in vitro and in vivo anti-leukemia activity of the stem bark of *S. impressifolia*. Although this species is of ethnopharmacological relevance to the treatment of several diseases (e.g., cancer treatment), scientific works have only investigated its antioxidant and chemical constituents.

Extract EAE, fractions FEAE.3 and FME.1, and quinonemethide triterpenes (22-hydroxytingenone, tingenone and pristimerin) in the stem bark of *S. impressifolia* exhibit potent cytotoxicity against a small panel of cancer cell lines representative of colon, breast, liver, tongue, gastric, skin and hematological cancers, including in vitro anti-leukemia activity against HL-60 and K-562 cells. According to our anticancer screening program, which complements the anticancer screening program of the National Cancer Institute of the United States of America, a crude extract with IC_{50} values of below 30 μg/mL and a lead compound with IC_{50} values of below 4 μg/mL in cancer cell line-based assays are considered promising as tools of anticancer drug development (Suffness and Pezzuto, 1990; Ribeiro et al., 2012; Bezerra et al., 2015; Silva et al., 2016; Dória et al., 2016). Extract EAE, fraction FEAE.3 and fraction FME.1 present IC_{50} values of below 30 μg/mL and quinonemethide triterpenes (22-hydroxytingenone, tingenone and pristimerin) present IC_{50} values of below 4 μg/mL, denoting their cytotoxicity. In particular, extract EAE, its fraction FEAE.3, and quinonemethide triterpenes 22-hydroxytingenone and tingenone exhibit the highest levels of cytotoxicity, and although they exhibit low levels of selectivity, they affect noncancerous cells when the power equivalent of the positive control is used (doxorubicin).

Like the cytotoxic effect observed for *S. impressifolia* extracts, methanolic aerial and root extracts of *S. oblonga* exhibit cytotoxic properties with IC_{50} values of 35 and 44 μg/mL for breast cancer cells (MDA-MB-231), respectively (Musini et al., 2015). In *S. impressifolia* extracts, this effect is associated with quinonemethide triterpenes 22-hydroxytingenone, tingenone and pristimerin, which also exhibit potent cytotoxicity. Tingenone and pristimerin are known to inhibit the cell growth of human myeloma RPMI8226 cells due to their ability to inhibit the polymerization of tubulin (Morita et al., 2008). Works on pristimerin also illustrate its capacity to exhibit cytotoxicity through the inhibition of the nuclear factor-κB signaling pathway in HCT-116 cells (Yousef et al., 2018), to induce caspase-dependent apoptosis in MDA-MB-231 cells (Wu et al., 2005) and to induce apoptosis through reactive oxygen species-mediated mitochondrial dysfunction in U87 glioma cells (Yan et al., 2013).

Extract EAE and its fraction FEAE.3 also inhibit the in vivo development of HL-60 cells engrafted in C.B-17 SCID mice. Pristimerin, a quinonemethide triterpene found in extract EAE, and its fraction FEAE.3 are also known to inhibit the in vivo development of HCT-116 cells engrafted in nude mice through the inhibition of NF-κB expression accompanied with the significant suppression of p65 phosphorylation p65 phosphorylation (Yousef et al., 2018). The inhibition of glioma growth in nude mice in vivo with subcutaneous xenograft glioma and the inhibition of a plasmacytoma grown from xenografted myeloma cells in nude mice has also previously been reported in relation to pristimerin treatment (Tiedemann et al., 2009; Zhao et al., 2016). Quinonemethide triterpenes 22-hydroxytingenone and tingenone, which are also found in extract EAE and in its fraction FEAE.3, have not previously been evaluated in an in vivo cancer xenograft model; however, they are potent cytotoxic agents and must contribute to the in vivo antileukemia activity of extract EAE and of its fraction FEAE.3.

Concerning the toxicity of these drugs, some unexpected effects were found in the drug-treated animals, including metastatic nodules in animals treated with fraction FEAE.3 and a reduction of body weight in animals treated with extract EAE and with its fraction FEAE.3, indicating toxicity. In our in vitro study we also observed the low selectivity of these drugs to cancer cells versus noncancerous cells. Thus, while these herbal drugs can suppress tumor growth in vivo, their toxicity may compromise their development as anticancer agents. On the other hand, currently used cancer chemotherapeutic drugs present severe levels of toxicity and cannot be applied to treat many forms of cancer such as acute myeloid leukemia. The development of new anticancer candidates is urgently needed, and even drugs with certain levels of toxicity can be tolerated. Moreover, no changes in organ weight were observed from our hematological analysis of peripheral blood in animals treated with extract EAE and with its fraction FEAE.3.

5. Conclusion

This study shows that ethyl acetate extract and its fraction from the stem bark of *S. impressifolia* exhibits in vitro and in vivo anti-leukemia activity that can be attributed to its quinonemethide triterpenes (22-hydroxytingenone, tingenone and pristimerin). These data confirm the ethnopharmacological use of this species and may contribute to the development of new anticancer herbal medicines.

CRediT authorship contribution statement

Ana Carolina B. da C. Rodrigues: Conceptualization, Methodology, Formal analysis. **Felipe P. de Oliveira:** Methodology, Formal analysis. **Rosane B. Dias:** Methodology, Formal analysis. **Caroline B.S. Sales:** Methodology, Formal analysis. **Clarissa A.G. Rocha:** Methodology, Formal analysis. **Milena B.P. Soares:** Methodology, Formal analysis. **Emmanoel V. Costa:** Methodology, Formal analysis. **Felipe M.A. da Silva:** Methodology, Formal analysis. **Waldireny C. Rocha:** Methodology. **Hector H.F. Koolen:** Conceptualization, Methodology, Formal analysis. **Daniel P. Bezerra:** Conceptualization, Methodology, Formal analysis, Writing - original draft.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.11.008.

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