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Article

Biological properties of *Schinus terebinthifolia* Raddi essential oil

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Schinus terebinthifolia Raddi green fruits essential oil (EO) was evaluated regarding its phytochemical profile, antimicrobial and cytotoxic activities, and toxicity. Gas chromatography with mass spectrometry was applied to identify its constituents, thereafter the minimum inhibitory concentration, minimum bactericidal and fungicidal concentrations, and its antibiofilm activity were evaluated. The EO cytotoxicity was assessed in tumor and non-tumor human cells, and *in vivo* toxicity was evaluated in a *Galleria mellonella* model. The major constituents of *S. terebinthifolia* EO were alpha-phellandrene and beta-phellandrene. The EO had a weak activity against all strains of *Candida albicans* (MIC 1000μg/mL) and had no activity against non-albicans strains, bacteria, and *C. albicans* biofilm. Cytostatic activity against all tumor cell lines was shown. Additionally, cell viability remained at EO concentrations up to 62.5 μg/mL. At 16 mg/mL, 50% hemolysis was observed, and it had low toxicity *in vivo*. Overall, the *S. terebinthifolia* EO was characterized by low antimicrobial and antibiofilm activities, with no evidence of toxicity to human tumor and non-tumor cells.

Keywords: Anacardiaceae. Medicinal plants. Products with antimicrobial action. Toxicity test.

INTRODUCTION

The oral cavity has an important complex and diverse microbiota. However, loss of homeostatic balance between microorganism and host may lead to infections that can compromise health locally or systemically, especially when associated with biofilms (Zhang, 2018). It is estimated that 65% of human infections derive from biofilms, including prevalent clinical conditions of the oral cavity (Scott *et al.*, 2007; De La Fuente-Núñez, Reffuveille, Fernández, 2013) such as oral candidiasis primarily caused by *Candida* genre of fungi (Swidergall, Filler, 2017) and conditions associated with bacteria, such as periodontal disease (Roberts, Darveau, 2015).

Pathogen resistance combined with a limited arsenal of antimicrobials (Łukaszuk, Krajewska-kułak, Kułak, 2017; Wang, Xu, Hsueh, 2016) has propelled the search for new compounds with good antimicrobial action, such as natural products that are sources of

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secondary metabolites (Newman, Cragg, 2016). One of these is *Schinus terebinthifolia* Raddi, a plant in the Anacardiaceae family that is commonly known in Brazil as *aroeira-da-praia, aroeira-vermelha,* and *pimenta-rosa* and as Brazilian pepper tree in English (Carvalho *et al*., 2013). *S. terebinthifolia* is frequently found as part of the caatinga, a vegetation biome that covers most of the Brazilian semiarid region. This plant is an important natural resource to be explored, because traditional communities living in these areas could economically and socially benefit from its production. Therefore, studies that encourage the use of *S. terebinthifolia* as a new source of income can help to improve the quality of life of these communities and enable their survival from natural resources in their own environment.

S. terebinthifolia has been used in folk medicine as a cicatricial and anti-inflammatory compound (Carvalho *et al*., 2013) in the treatment of oral conditions (Araujo *et al*., 2018) and as an antimicrobial agent (Gilbert, Favoreto, 2011; Do Nascimento *et al*., 2012). Traditional communities have used *S. terebinthifolia* as an extract (obtained from the inner bark, leaves, and fruits) and as an essential oil (EO) (from the leaves and fruit) (Gilbert, Favoreto, 2011). Recent scientific studies have investigated its properties such as antitumor and antioxidant effects (Gilbert, Favoreto, 2011; Bendaoud *et al*., 2010), anti-inflammatory effects (Estevão *et al*., 2017), and antimicrobial activity (Dannenberg *et al*., 2019; Oliveira *et al*., 2018).

Most of these published articles used *S. terebinthifolia* EO obtained from ripe fruit (Bendaoud *et al*., 2010, Dannenberg *et al*., 2019; Oliveira *et al*., 2018); thus, additional studies are required to assess whether *S. terebinthifolia* EO obtained from green fruit is an effective and safe source of active compounds. This was the aim of the present study, since previous investigations have already indicated differences in the chemical composition of green and ripe fruits of *S. terebinthifolia* (Barbosa, Demuner, Clemente, 2007; Do Nascimento *et al*., 2012; Ennigrou *et al*., 2017). Moreover, it is known that different proportions of chemical constituents might give rise to different pharmacological effects. No study has analyzed the EO obtained from *S. terebinthifolia* green fruit collected from the caatinga biome of Brazil. Therefore, the present study aimed to assess the chemical composition of the EO of *S. terebinthifolia* green fruit and to evaluate its antimicrobial activity, cytotoxicity, and systemic toxicity.

MATERIAL AND METHODS

Plant material

Green fruits of *S. terebinthifolia* were collected in the semi-arid region of Campina Grande, Paraíba, Brazil (7º12 '35 "S, 35º 54' 57" W). The specimens were deposited in the collection of the Herbarium Manuel de Arruda Câmara (ACAM), State University of Paraiba (UEPB), Campus I, Campina Grande, Paraíba, under registry number 486/ACAM.

Essential oil

Hydrodistillation was performed in a Clevenger type system, at a proportion of 1:4 (m/v), with a distillation time of 2 h.

Essential oil fractions

Chemical compounds were initially monitored by thin layer chromatography (TLC) with silica gel matrix (TLC silica gel 60 F254 – Merck® - Darmstadt, Germany). A mixture of ethyl acetate (Synth® - São Paulo, Brazil) and hexanoic acid (Synth® - São Paulo, Brazil) (85:15 v/v) was used as mobile phase. The components were revealed using anisaldehyde chromate. Fractionation of the EO was carried out on solid-liquid silica phase (Merck® - Darmstadt, Germany) with a porous plate funnel-filter chromatographic column according to the gradient of polarity: hexane (1x100mL), hexane:ethyl acetate ratio of 98:2, 96:4, 96:4/92:8, 90:10, 88:12, 86:14, 84:16, 82:18, and 80:20 (v/v). Fractions were monitored by TLC and pooled according to their similarity profile and then concentrated under vacuum (Rotavapor R-215, BUCHI® - São Paulo, Brazil). The result was six final fractions that were tested for their antiproliferative activity against human tumor cell lines

Phytochemical characterization

The EO was chemically identified using a gas chromatograph (GC) (QP 2010 Plus, Shimadzu Co, São Paulo, Brazil) coupled to a mass spectrometer (MS) equipped with a DB-5 capillary column (30m x 0.25µm x 2.5m) (J&W Scientific – California, USA) and a scanning mode detector (40 – 400 m/z). The EO (400 µL) was inserted into glass vials, and 1.0 mL of trimethylsilyl for silanization solution were added. The temperature program was 60°C (0.3 min), followed by 240°C (15 min), at an increase of 3°C/min. The sample (0.5 µL) was injected by an auto-injector on the split-less mode, and integration was performed using a specific software from the equipment. The analytes were identified by comparison using the database of the equipment (NIST library), and the literature (Adams, 2007). Additionally, it was conducted a comparison with the data obtained from GC-MS (retention index and fragmentation time) of authentic patterns under the same conditions.

Evaluation of antimicrobial activity

The minimum inhibitory concentration (MIC), bactericidal concentration (MBC) and fungicidal concentration (MFC) (CLSI, 2008; CLSI, 2009) were obtained for the following microorganisms: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 11775), *Salmonella enteritidis* (ATCC 13076), *Candida parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 90030, *C. krusei* ATCC 34135, *C. albicans* (ATCC 10231), *C. albicans* (ATCC 5314), and two clinical strains of *C. albicans* obtained from oral candidiasis lesions from volunteers. All ethical aspects were respected (process number: 51779315.7.0000.5187).

The microdilution method was applied. Bacteria were grown in Müller-Hinton broth (Kasvi® Paraná, Brazil) and yeast were grown in RPMI 1640 (Sigma-Aldrich® - Missouri, EUA). The EO serially was diluted (2000 to 0.4882µg/mL) in the plates wells. Subsequently, bacterial (5×10^5 colony forming units - (CFU)/mL) and fungal (2.5×10^3 CFU/mL) suspensions were added and the plates were incubated at 37°C for 24 h. Chloramphenicol and nystatin (both 500µg/mL) (Sigma-Aldrich®) were used as pharmacological controls. Visible microbial growth was confirmed with triphenyl tetrazolium chloride for bacteria and by the changing coloration of the RPMI 1640 medium for yeast. After 24 h, 50µL of each well, with equal and/or higher MICs were sub-cultured (37ºC, 24h) on brain heart infusion agar (Sigma-Aldrich®) (bacteria) or sabouraud dextrose agar (Kasvi®) (yeast).

Inhibition of *Candida albicans* biofilm

The biofilm assay was performed with the previously stated *C. albicans* strains. *C. albicans* suspensions (1×10^7CFU/mL) were prepared in RPMI 1640 (Sigma-Aldrich®) and 100µL was distributed to each well of a 96-well microplate and incubated at 37°C for 24 h. The biofilms were then exposed to EO at concentrations based on the MIC (1000 µg MIC, 2000 µg 2×MIC, and 4000 µg 4×MIC). Nystatin was used as a control. After treatment, the biofilms were seeded onto saboraud dextrose agar plates (Kasvi®) to assess the number of viable microorganisms (Silva *et al*, 2019b).

In vitro antiproliferative activity evaluation

The antiproliferative activity was evaluated against a panel of eight human tumor cell lines [U251 (glioblastoma), MCF-7 (breast, adenocarcinoma), NCI-ADR/RES (multi-drug resistant ovarian adenocarcinoma), 786-0 (kidney, adenocarcinoma), NCI-H460 (lung, large cell carcinoma), PC3 (prostate, adenocarcinoma), HT-29 (colon, adenocarcinoma), and K562 (chronic myeloid leukemia)] and one non-tumor human cell line (HaCaT, immortalized keratinocytes). The tumor cell lines were kindly provided by the Frederick Cancer Research & Development Center, National Cancer Institute, Frederick, MA, USA. The non-tumor cell line was provided by Dr. Ricardo Della Coletta (University of Campinas). Stock cultures were grown in 5 mL of complete medium [RPMI-1640 (Gibco, USA)] supplemented with 5% fetal bovine serum (FBS, Gibco, USA, catalog number 16000044) and 1% penicillin–streptomycin mixture [1000 U·mL-1:1000 µg·mL-1 (Vitrocell, Brazil)] at 37ºC in a 5% CO_2 humidified atmosphere. All experiments were conducted with cells grown for 5 to 12 passages.

The EO and its fractions were first diluted to 100 mg/mL in DMSO (Merck - Darmstadt, Germany), followed by serial dilution in complete RPMI 1640 medium, affording the final EO concentrations of 0.25, 2.5, 25, and 250 µg/mL. Twenty-four hours after being transferred to 96-well plates (inoculation density: 3 to 6 x 10^4 cell/mL, 100 µL/well), all cell lines were exposed to freshly diluted EO samples, in triplicate (100 µL/well), for 48 h at 37 °C under 5% CO_2. Doxorubicin (0.025, 0.25, 2.5, and 25 µg/mL, 100 µL/well) was used as a positive control. Before the 48 h exposure, one control plate (T_0) containing cells for each tested cell line was utilized for cell fixation using trichloroacetic acid (TCA, 50%, 50 µL/well) (Sigma-Aldrich) to establish the initial cell amount. After the 48 h exposure, treated cells were fixed with 50% TCA for 1 h at 4°C and then washed and kept at room temperature until completely dry. The cellular protein content was then stained with sulforhodamine B dye (0.4% in 1% acetic acid, 50 µL/well) (Sigma-Aldrich), and the bound dye was solubilized with Trizma base solution (10 µM, pH 10.5, 150 µL/well) (Sigma-Aldrich). The spectrophotometric reading was performed at 540 nm with a VersaMax plate reader (Molecular Devices).

Based on the cell absorbance before (T_0) and after (T1) EO exposure, the cell growth (%) for each tested EO sample against each cell line was calculated by the following equations: $100\times[(T\text{-}T_0)/(T1\text{-}T_0)]$ when $T > T_0$; and $100\times[(T\text{-}T_0)/T_0)]$ when $T \leq T_0$. T is the mean absorbance of treated cells, T1 is the mean absorbance of untreated cells after 48 h of exposure, and T_0 is the mean absorbance of untreated cells at the beginning of the 48 h exposure (Monks *et al.*, 1991; Shoemaker, 2006). Based on these results, the sample concentration required to achieve total growth inhibition (TGI) was calculated by sigmoidal regression using Origin 8.0 software.

The selectivity index (SI) was calculated according to Eq. (1).

$$SI^{cancer\ line}_{HaCat}{}_{(compound)} = \frac{GI_{50}(HaCat)}{GI_{50}(cancer\ line)} \quad (1)$$

Cytotoxicity against RAW 264.7 macrophage

A suspension of RAW 264.7 macrophages ($1x10^5$ cells/mL) (ATTC, Manassa, VA, USA) was prepared in RPMI 1640 (Sigma-Aldrich®) containing 5% FBS (Gibco® BRL – Dublin, Ireland) and 2 mg/mL penicillin-streptomycin (Sigma-Aldrich®). Aliquots of 100 µl of suspension were applied to each well of the 96-well plates and incubated for 24 h at 37° C under 5% CO_2, a control plate was also prepared (T_0 plate). The EO was initially diluted in DMSO, followed by serial dilution on RPMI 1640 (250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.49, and 0.24 µg/mL) (T-plate). The plates were incubated for 48 h at 37°C in 5% CO_2.

Previous to EO sample addition, the cells in the T_0 plate were fixed with 50% TCA (50 µL/well) to determine the cell quantity at the time of EO addition. After 48 h, the T-plate cells were fixed with 50% TCA and incubated for 1 hour, followed by washing with distilled water to remove any residue. After the plates were dried at room temperature, 50 µL of 0.4% sulforhodamine B protein (SRB) diluted in 1% acetic acid as added to the wells. The plates were held at room temperature for 30 minutes and then washed 4 times with 1% acetic acid to remove the dye that had not attached to the cells. Dye that had bound to the cellular proteins was solubilized with 150 µL/well Trizma base solution (10 µM, pH 10.5). The absorbance was read at 540 nm (VersaMax, Molecular Devices), and viability was calculated by the following equation: cell viability (%) = (sample/control) x 100, where sample and control are equivalent to the absorbance of the wells with and without the addition of treatment, respectively.

In vitro toxicity against human erythrocytes

The hemolysis assay was performed as previously described (Luize *et al.*, 2005). A 5% suspension of erythrocytes was mixed with different concentrations of the EO (16, 8, 4, 2 and 1 mg/mL). After incubating for 1 h at room temperature, the samples were centrifuged (500 g, 10 min), and the absorbance at 540 nm was read by a spectrophotometer (UV mini – 1240 - Shimadzu® - São Paulo, Brazil). The level of hemolysis was scored as follows: (–) 0% hemolysis; (+) 1 - 25% hemolysis;

(++) 26 - 50% hemolysis; (+++) 51 - 75% hemolysis; and (++++) 76 - 100% hemolysis. A saline-treated erythrocyte suspension was used as a negative control. Turk's liquid (2% acetic acid) plus methylene blue was used as a positive control.

Systemic toxicity test in *Galleria mellonella* model

The EO was evaluated *in vivo* to assess its acute systemic toxicity using the *Galleria mellonella* larvae model (Rochelle *et al.*, 2016). Doses of the EO (50 to 10g/kg) in were used to determine the LD_{50}. Ten larvae were randomly selected for each group. 5 µL of each EO concentration diluted in 20% DMSO (Merck® - Darmstadt, Germany) was added to test and control groups. The larvae were incubated at 30°C, and their survival was evaluated every 9 h up to 48 h. The larvae that did not move and that presented melanization were considered as dead.

Statistical analysis

Analyses were performed in triplicate and in three independent experiments. The data were initially evaluated to determine their distribution using the Kolmogorov - Smirnov test. One way analysis of variance (ANOVA) and the post-hoc Tukey test were applied at a significance level of 5% ($\alpha<0.05$).

RESULTS AND DISCUSSION

In accordance with Holetz *et al.* (2002), the EO presented weak activity against all strains of *C. albicans* (MIC 1000 µg/mL), and no evidence was observed of growth inhibition for the non-albicans strains, or for the bacterial species tested (MIC > 1000 µg/mL) (Table I). Moura *et al.* (2011) found limited antimicrobial activity for *S. terebinthifolia*, similar to the findings of the present study. In the Moura study, the authors analyzed a gel containing *S. terebinthifolia* extract against *Candida* and bacteria species, and no significant decrease in the microbial load was observed.

In contrast to the findings of the current study, favorable antimicrobial results were reported for the EO obtained from *S. terebinthifolia* ripe fruits against fungus (Oliveira *et al.*, 2018) and gram-positive and gram-negative bacteria (Dannenberg *et al.*, 2019). The differing results can be attributed to differences in the chemical composition between ripe and green fruits, which was demonstrated by Ennigrou *et al.* (2017). The authors concluded that the plant maturation process resulted in a significant change in its chemical composition. They stated that these changes were reflected in the antimicrobial activity such that gram-positive and gram-negative bacteria were more susceptible to the ripe fruit EO than to the green fruit EO.

TABLE I - Minimum Inhibitory Concentration (MIC), Minimum Fungicidal (MFC) and Minimum Bactericidal (MBC) concentrations of the *S. terebinthifolia* green fruits EO, according to different microbial species

MICROORGANISMS	*S. terebintifolius* essential oil			CONTROLS	
	MIC (µg/mL)	MFC or MBC (µg/mL)	MFC/MIC ratio	Nystatin MIC (µg/mL)	Chloramphenicol MIC (µg/mL)
Candida albicans ATCC 10231	1000	1000	1	0.9765	-
Candida albicans MYA 2876	1000	1000	1	0.9765	-
Candida albicans - Clinical strain A2	1000	1000	1	1.9531	-
Candida albicans - Clinical strain A8	1000	1000	1	1.9531	-
Candida krusei	>2000	>2000	-	0.9765	-

TABLE I - Minimum Inhibitory Concentration (MIC), Minimum Fungicidal (MFC) and Minimum Bactericidal (MBC) concentrations of the *S. terebinthifolia* green fruits EO, according to different microbial species

MICROORGANISMS	***S. terebintifolius* essential oil**			**CONTROLS**	
	MIC (µg/mL)	**MFC or MBC (µg/mL)**	**MFC/MIC ratio**	**Nystatin MIC (µg/mL)**	**Chloramphenicol MIC (µg/mL)**
Candida dubliniensis	2000	>2000	-	1.9531	-
Candida glabrata	2000	>2000	-	3.9062	-
Streptococcus. aureus	>1000	-	-	-	8
Escherichia coli	>1000	-	-	-	8
Salmonella enteritidis	>1000	-	-	-	125

MIC: Minimum Inhibitory Concentration
MBC: Minimum Bactericidal Concentration
MFC: Minimum Fungicidal Concentration
Fungicide (MFC / MIC <4) or fungistatic (MFC / MIC> 4) (SIDDIQUI et al., 2013).
* (-) Could not determine.

C. albicans is a susceptible yeast when in a planktonic form. However, in the biofilm assay, *C. albicans* MYA 2876 and the two clinical strains showed no reduced viability (expressed in CFU) upon EO exposure. A small but significant reduction was seen in the viability of *C. albicans* ATCC 10231 (Figure 1). These results may be associated with the limited penetration of the *S. terebinthifolia* EO into the biofilm structure. Biofilm formation is considered one of the main virulence factors associated with *C. albicans*. Additionally, *c*ells within the biofilms are protected from environmental stresses including host immune defenses and antifungal treatment, which carries important clinical consequences for the treatment of biofilm-associated infections (Wall *et al*., 2019).

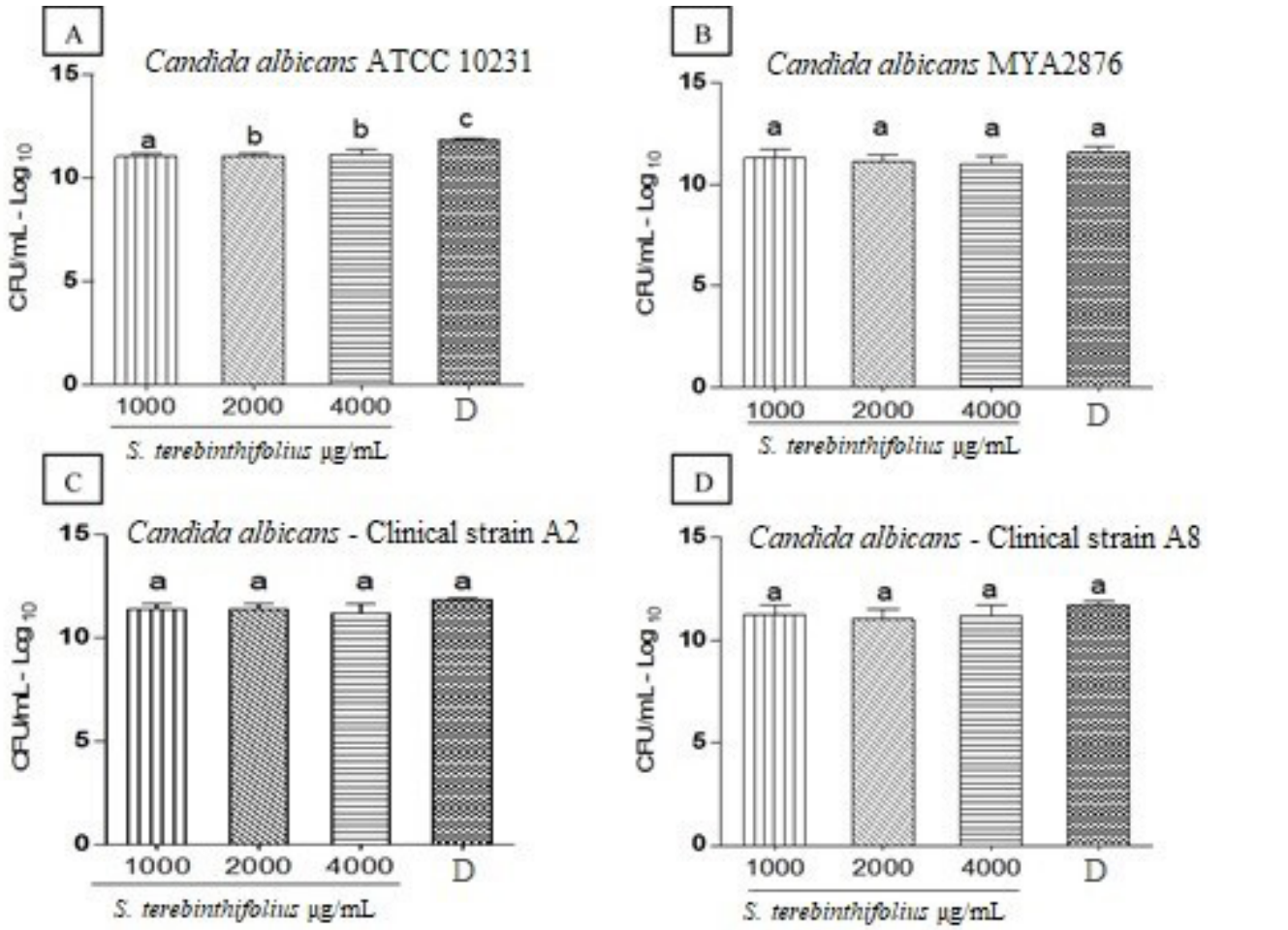

FIGURE 1 - Anti-biofilm activity of *S. terebinthifolia* green fruits EO at concentrations 1000 µg/mL (MIC), 2000 µg/mL (twice MIC) and 4000 µg/mL (four times MIC) on *C. albicans* strains.

The EO extracted from *S. terebinthifolia* green fruits had a yield of 1 mL. Its chemical composition is presented in Table II. The major constituints of *S. terebinthifolia* green fruits EO are diterpenes: alpha-feladrene (37.05%), beta-feladrene (24.10%), alpha-pinene (15.91%) and germacrene D (14.47%). Do Nascimento *et al*. (2012) analyzed the chemical composition of *S. terebinthifolia* green fruit EO, and alpha-phellandrene was one of the major compounds identified, but in a different proportion from that identified in the current study. Furthermore, unlike the present study, other compounds were previously identified, such as limonene and beta-pinene. These differences might be associated with changes in the conditions to which the plants were subjected; Do Nascimento *et al.* (2012) collected plants from a region of Atlantic rainforest in the northeast of Brazil, which is a different biome from caatinga. Previous studies have also indicated a seasonality effect in the chemical compositions of natural compounds (Macedo *et al*., 2020; Bitu *et al*., 2015).

Ennigrou *et al*. (2017) compared the chemical compositions of *S. terebinthifolia* EO obtained from green and ripe fruits. The authors found alpha-phellandrene, alpha-pinene, and germacrene D among the major compounds in the green fruit EO, similar to the present findings. Additionally, the authors concluded that a significant change was seen in the chemical profile of

the *S. terebinthifolia* fruit after the maturation process. Para-cymene and germacrene D were more abundant in the EO obtained from green fruit, while a sharp decrease in alpha-pinene and beta-phellandrene was noted in the ripe fruit EO. The differing chemical compositions of ripe and green fruit EO might explain the different biological activities of the EOs. Indeed, the present study was performed to investigate this issue, because most of the previously published articles used EO extracted from ripe fruit.

TABLE II - Analyses identified in the essential oil of *S. terebinthifolia* green fruits EO*,* by gas chromatography with mass spectrometry

T_R (MIN)	RI [A]	COMPONENT	% REL. [B]
5.61	933	alpha-pinene	15.91
7.10	991	beta-myrene	1.07
7.57	1007	alpha-phellandrene	37.05
8.18	1025	para-cymene	5.35
8.35	1029	beta-phellandrene	24.10
17.02	1252	n.i. (c)	2.05
26.43	1481	germacrene D	14.47

Notes: a) Relative retention index; b) Fraction in percentage of the total integrated area for the chromatogram; c) Not identified.

The antiproliferative activity (Table III, Figure 2) was assessed using the protocol developed by the Frederick Cancer Research & Development Center, National Cancer Institute (Shoemaker, 2006) using doxorubicin (Table III, Figure 2A) as a positive control. According to Fouche *et al.* (2008), the antiproliferative activity of compounds can be classified as inactive (TGI ≥ 50 μg/mL), weak (15 μg/mL ≤ TGI < 50 μg/mL), moderate (6.25 μg/mL ≤ TGI < 15 μg/mL), or potent (TGI < 6.25 μg/mL). Based on the concept of the therapeutic index described by Muller and Milton (2012), we calculated the selective index (SI) by determining the ratio between the TGI value for a non-tumor cell line (HaCaT, immortalized human keratinocytes) and the TGI value for each tumor cell line. The SI indicates the potential antiproliferative effect against normal tissues such as mucosa and bone marrow (i.e., an on-target toxicology approach).

Using these criteria, the *S. terebinthifolia* EO (Table III, Figure 2B) and the hexane:ethyl acetate fraction 96:4 (Table III, Figure 2C) were considered as inactive, showing nonspecific cell death up to 250 μg/mL. The most active fraction, the hexane:ethyl acetate fraction 80:20, exhibited potent activity (TGI < 0.25 μg/mL, Table III, Figure 2D) against almost all tumor cell lines and against the non-tumor cell line (HaCaT).

The hexane:ethyl acetate fraction 90:10 (Table III, Figure 2E) showed potent and selective cytostatic effects against leukemia (K562, TGI = 1.3 μg/mL, SI = 7.2), glioblastoma (U251, TGI = 1.4 μg/mL, SI = 6.5), kidney (786-0, TGI = 2.0 μg/mL, SI = 4.6), and prostate cell lines (PC-3, TGI = 4.2 μg/mL, SI = 2.2). The hexane:ethyl acetate fraction 86:14 (Table III, Figure 2F) was as potent as the hexane:ethyl acetate fraction 90:10 against the leukemia (K562, TGI = 1.4 μg/mL, SI = 9.4) cell line despite being less active than the hexane:ethyl acetate fraction 90:10 against U251, 786-0, and PC-3 cells. Both the hexane:ethyl acetate fractions 90:10 and 86:14 showed moderate to weak cytostatic effects against the other tumor cell lines (Table III).

Further, the hexane:ethyl acetate fraction 94:6/92:8 (Table III, Figure 2G) showed weak and low selective cytostatic effects against glioblastoma (U251, TGI = 42.7

μg/mL, SI = 1.5), prostate (PC-3, TGI = 43.2 μg/mL, SI = 1.5), and leukemia tumor cell lines (K562, TGI = 48.1 μg/mL, SI = 1.3). Finally, the hexane:ethyl acetate 98:2 fraction (Table III, Figure 2H) showed cytostatic effects ranging from potent (K562 and 786-0) to moderate (U251) and weak (MCF-7, NCI-ADR/RES, and HT29), while it was inactive against the HCI-H460 and PC-3 cell lines.

Previous studies have demonstrated the antiproliferative effect of *S. terebinthifolia* EO obtained from ripe fruits (Bendaoud *et al*., 2010; Silva *et al*., 2017; Silva *et al*., 2019a).

Silva *et al*. (2017) and Silva *et al*. (2019a) used different methods of extraction (supercritical CO_2 extraction and optimized hydrodistillation using a Clevenger apparatus, respectively), and they identified similar compounds as in the current study of green fruit EO, such as alpha-phellandrene, alpha-pinene, and germacrene D, but in different proportions. The EO in Silva *et al*. (2019a) showed potent activity for all cell lines investigated, with promising results against leukemia, kidney, multidrug-resistant ovarian, and prostate tumor cell lines. Silva *et al*. (2017) found potent activity against multidrug-resistant ovarian, prostate, and ovarian tumor cell lines. Both studies discussed the possible association between the chemical compounds identified in the EO and the antiproliferative activity.

Bendaoud *et al*. (2010) demonstrated an antiproliferative effect of the EO against human breast cancer cells (MCF-7). The authors found alpha-phellandrene, alpha-pinene, and germacrene D as some of the major compounds in the EO, similar to this study. The authors attributed the antiproliferative activity of the EO to the presence of sesquiterpenes, such as germacrene D. Additionally, other compounds present in the unripe fruit EO analyzed in the present study have been associated with antiproliferative effects. For example, alpha-phellandrene has been described as inducing tumor cell necrosis through ATP depletion in human tumor liver cells (Hsieh *et al*., 2014), and alpha-pinene (the third-most abundant compound identified in the present study) has been described as an apoptosis inductor in malignant melanoma cells (Matsuo *et al*., 2011).

TABLE III - Antiproliferative activity *S. terebinthifolia* green fruits EO and its fractions against human tumor and non-tumor cell lines expressed as concentration required to elicit total growth inhibition (TGI, μg/ml)

Cell lines	Doxorrubicin		*S. terebinthifolia* EO		98:2		96:4		94:6/92:8		90:10		86:14		80:20	
	TGI	SI	TGI[a]	SI[b]	TGI	SI	TGI	SI	TGI	SI	TGI	SI	TGI	SI	TGI	SI
U251	0.98 ± 0.66	0.3	115.9 ± 40.1	2.2	6.4 ± 4.4	4.4	82.8 ± 3.7	2.6	42.7 ± 2.2	1.5	1.4 ± 1.0	6.5	4.2± 1.6	3.2	< 0.25	n.c.
MCF-7	5.7 ± 6.2	0.1	76.6 ± 17.5	3.3	27.9 ± 5.0	1.0	226.0 ± 0.3	1.0	52.9 ± 5.4	1.2	6.0 ± 1.8	1.5	6.4 ± 2.3	2.1	0.33 ± 0.16	n.c.
NCI-ADR/RES	13.0 ± 7.5	0.03	108.8 ± 53.5	2.3	24.3 ± 7.7	1.2	122.8 ± 26.4	1.8	63.0 ± 15.5	1.0	17.9 ± 9.4	0.5	10.2 ± 4.0	1.3	< 0.25	n.c.
786-0	0.51 ± 0.23	0.7	250*	1.2	5.0 ± 5.8	5.6	110.7 ± 8.9	2.0	58.8 ± 6.7	1.1	2.0 ± 1.4	4.6	5.4 ± 0.5	2.5	< 0.25	n.c.
NCI-H460	0.13 ± 0.17	2.6	105.9 ± 25.3	2.4	91.7 ± 14.6	0.3	103.8 ± 28.2	2.1	90.0 ± 43.5	0.7	12.9 ± 5.5	0.7	9.7 ± 5.3	1.4	8.5 ± 3.3	n.c.
PC-3	1.3 ± 0.7	0.3	76.4 ± 29.2	3.3	> 250	0.1	85.8 ± 0.1	2.5	43.2 ± 3.9	1.5	4.2 ± 0.7	2.2	5.7 ± 1;6	2.3	< 0.25	n.c.
HT-29	9.2 ± 9.1	0.04	94.1 ± 13.3	2.7	40.4 ± 21.1	0.7	224.8 ± 0.5	1.0	73.81 ± 2.4	0.9	8.3 ± 2.7	1.1	8.3 ± 4.0	1.6	0.25*	n.c.
K562	2.8 ± 2.3	0.1	250*	1.2	2.4 ± 1.3	11.8	110.6 ± 3.0	2.0	48.1 ± 6.5	1.3	1.3 ± 0.4	7.2	1.4 ± 0.8	9.4	< 0.25	n.c.
HaCaT	0.34 ± 0.05	n.a.	250*	n.a.	28.2 ± 2.5	n.a.	217.6± 0.6	n.a.	63.4 ± 0.5	n.a.	9.2 ±3.1	n.a.	13.3 ± 6.3	n.a.	< 0.25	n.a.

a) TGI values expressed in μg/ml followed by standard error, calculated by sigmoidal regression using Origin 8.0 software; b) SI: selectivity index calculated as $GI_{50\ HaCaT}$/ $GI_{50\ Tumor\ cell\ line}$; *approximated value (experimental data did not converge); n.c.: not calculated; n.a.: not applied.

Human tumor cell lines: U251 (glioblastoma); MCF-7 (breast, adenocarcinoma); NCI-ADR/RES (ovary, multi-drug resistant adenocarcinoma); 786-0 (kidney, adenocarcinoma); NCI-H460 (lung, large cell carcinoma), PC-3 (prostate, adenocarcinoma), HT-29 (colon, adenocarcinoma), K562 (chronic myeloid leukemia). Human non-tumor cell lines: HaCaT (immortalized keratinocyte)

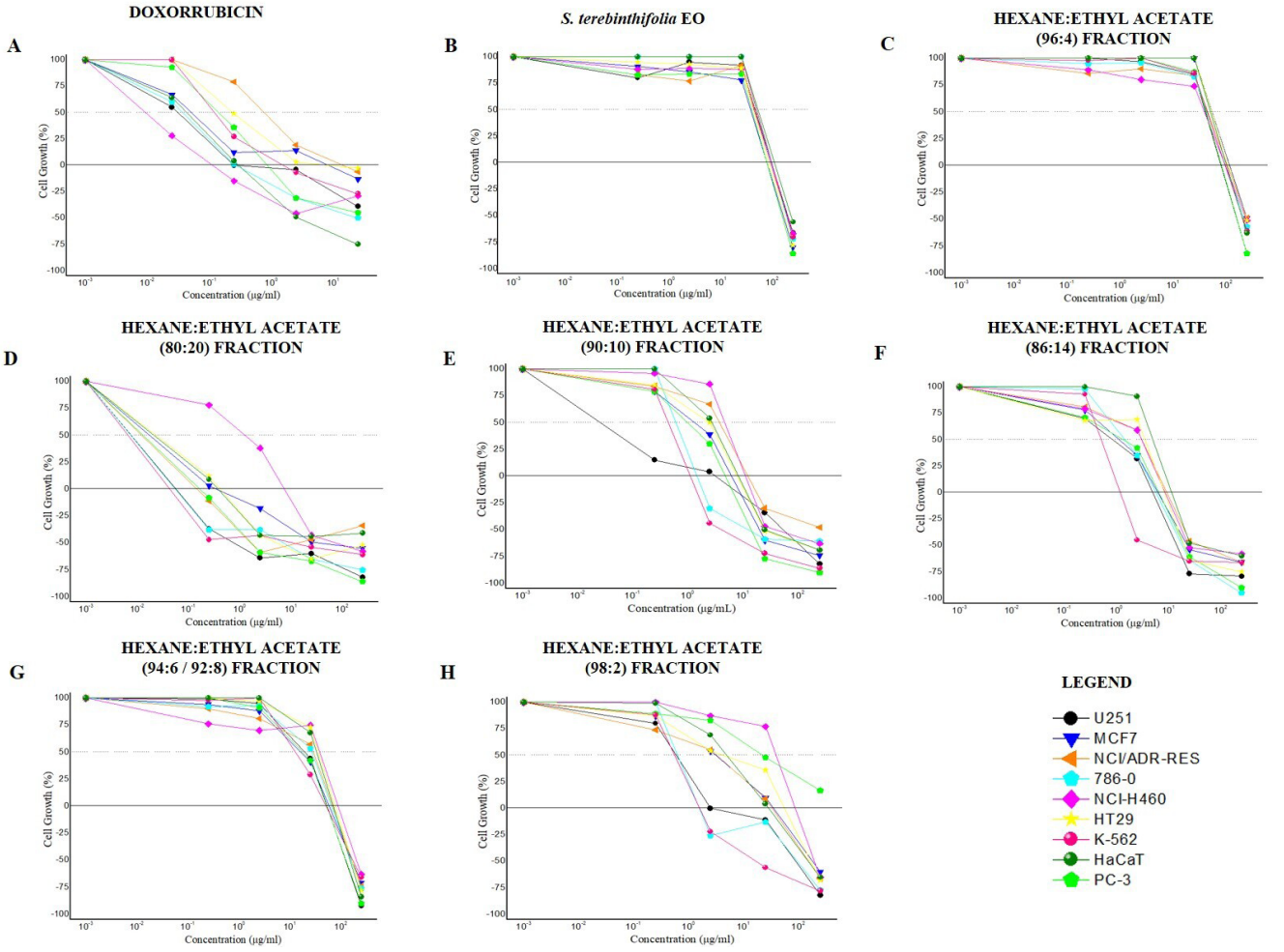

FIGURE 2 - Cell lines proliferation according to the concentration of positive control Doxorubicin (Figure 2A), *S. terebinthifolia* green fruits EO (Figure 2B) and its hexane: ethyl acetate fractions (Figure 2C-2H).

The hemolysis test, which showed 50% hemolysis at 16 mg/mL. (Table IV), and the antiproliferative activity against keratinocytes represent initial safety data for the use of this EO. Erythrocyte membrane stability is assessed in toxicity screening and is an indicator of *in vitro* damage, especially when associated with oxidative stress (Pathak, Sharma, Shrivastva, 2012). The results from the *Galleria mellonella* larvae model suggest a good safety profile, since only high doses (40g/kg) affected the larvae viability, resulting in a 10% death rate (Figure 3). This methodology has been gaining in prominence due to its applicability in toxicological testing and the efficacy for applying it to new compounds (Freires *et al.*, 2017).

TABLE IV - Percentage distribution of the hemolysis produced by the *S. terebinthifolia* green fruits EO, according to the concentrations

CLASSIFICATION OF HEMOLYSIS						
Concentration		**16 mg/mL**	**8 mg/mL**	**4 mg/mL**	**2 mg/mL**	**1 mg/mL**
S. terebinthifolia	% hemolysis	53%	46%	12.38%	11.26%	5.84%
		++	+	-	-	-

(–) 0% hemolysis; (+) 1-25% hemolysis; (++) 26-50% hemolysis; (+++) 51-75% hemolysis; and (++++) 76-100% hemolysis.
Positeve control (0%): Saline-treated erythrocyte suspension
Negative control (100%): Turk's liquid plus methylene blue

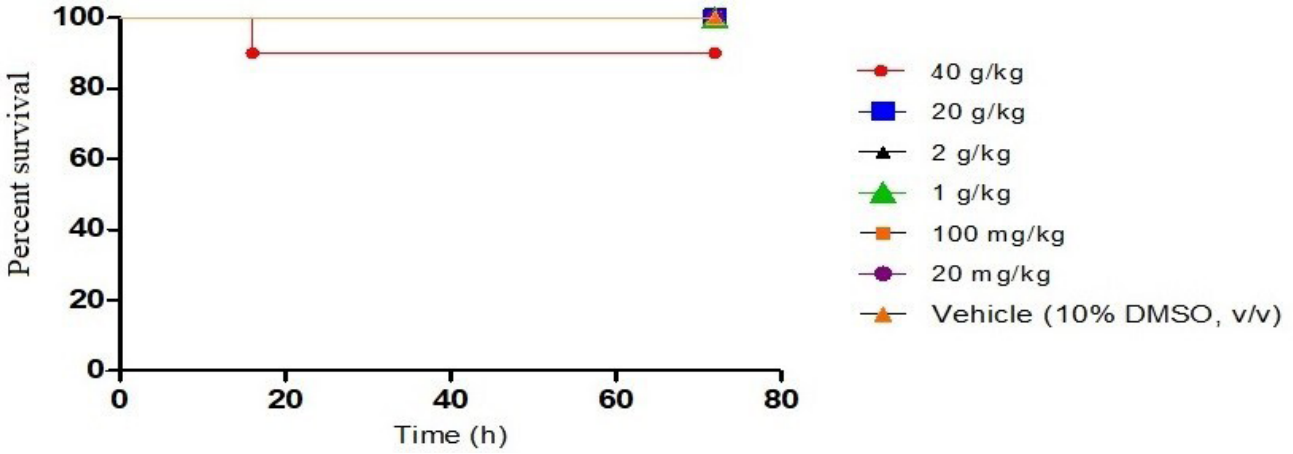

FIGURE 3 - *In vivo* toxicity of *S. terebinthifolia* green fruits EO in invertebrate *Galleria mellonella* model.

According to the International Organization for Standardization (ISO) publication 10993-5 (ISO, 2009), concentrations that reduce cell viability to less than 70% are considered cytotoxic. *S. terebinthifolia* green fruits EO resulted in high cell viability grater than 85% for RAW 264.7 macrophages exposed to concentrations of 62.5µg/mL (Figure 4). These macrophage viability results could guide future studies to evaluate the anti-inflammatory potential of this EO.

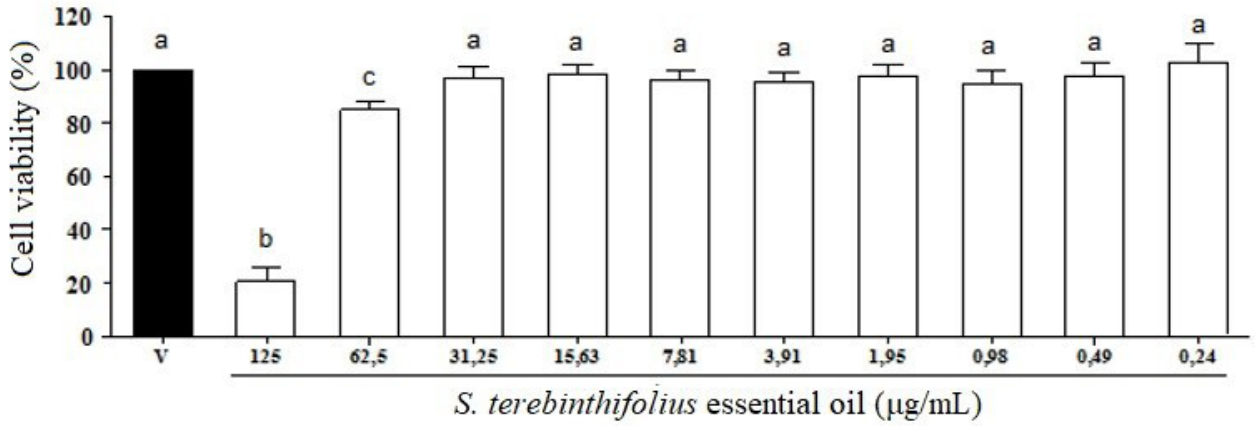

FIGURE 4 - Cell viability percentage of RAW 264.7 macrophages lineage according to the concentration of S. terebinthifolia green fruits EO.

Although *S. terebinthifolia* is as an antimicrobial agent in folk medicine (Do Nascimento *et al*., 2012), the results obtained in the present study showed low antimicrobial and antibiofilm activity. Therefore, additional studies should be conducted to further investigate its use as an effective compound against microorganisms of clinical relevance. However, it presented low cytotoxic potential as demonstrated in the *in vitro* and *in vivo* models. Considering these results and the phytochemical characteristics of this EO, other properties could be considered, such as anti-inflammatory activity (Estevão *et al*., 2017). Furthermore, additional *in vitro* and *in vivo* assays are needed to assess pharmacological efficacy.

CONCLUSION

The EO from *S. terebinthifolia* green fruits showed low antimicrobial and antibiofilm activity. The major compounds identified were diterpenes, including alpha-phellandrene, beta-phellandrene, alpha-pinene, and germacrene D. Some EO fractions showed moderate antiproliferation activity against tumor cell lines, and no evidence of cytotoxic activity was identified.

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