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Anatomical aspects, chemical analysis and cytotoxic effect of the essential oil from leaves of *Casearia arborea* (Salicaceae)

[Aspectos anatómicos, análisis químico y efecto citotóxico del aceite esencial de *Casearia arborea* (Salicaceae)]

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Abstract: The genus Casearia (Salicaceae) is found in sub-tropical and tropical regions of the world and comprises about 200 species. In Brazil, there are about 48 species and 12 are registered in the State of Rio de Janeiro; including *Casearia arborea* (Rich.) Urb. Essential oil was obtained from the fresh leaves by hydrodistillation and analyzed by GC-MS and GC-FID. The cytotoxic effect was determined by WST-1 assay. Chemical analysis of the essential oil revealed a very diversified (n = 37 compounds) volatile fraction composed mainly of non-oxygenated sesquiterpenes (90.2%). These sesquiterpenes included byciclogermacrene (18.7%), germacrene D (12.1%) and α-humulene (11.5%). In addition, the essential oil demonstrated cytotoxic effects against A549 tumor cells in the concentration of 4 µg/mL (EC_{50}) ($p < 0.05$).

Keywords: A549, byciclogermacrene, *Casearia arborea*, salicaceae, secretory cavities, sesquiterpene.

Resumen: El género Casearia (Salicáceas) se encuentra en las regiones tropicales y sub-tropicales del planeta y comprende alrededor de 200 especies. En Brasil existen 48 especies, 12 de las cuales fueron registradas en el Estado de Río de Janeiro incluyendo *Casearia arborea* (Rich.) Urb. El aceite esencial fue extraído de hojas frescas por hidrodestilación y analizado por GC-MS y GC-FID. El efecto citotóxico fue determinado por ensayo WST-1. Las cavidades secretorias fueron ocasionalmente encontradas tanto en la lámina foliar como en el pecíolo. El análisis químico del aceite esencial reveló una muy diversa fracción volátil (n = 37 compuestos) formada principalmente por sesquiterpenos no oxigenados (90,2%). Estos sesquiterpenos incluyen biciclogermacreno (18,7%), germacreno D (12,1%) y α-humuleno (11,5%). Además, el aceite esencial demostró efectos citotóxicos contra las células tumorales A549 en una concentración de 4µg/mL (EC_{50}) ($p < 0.05$).

Palabras clave: A549, biciclogermacreno, *Casearia arborea*, cavidades secretorias, salicáceas, sesquiterpenos.

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INTRODUCTION

Casearia genus is distributed in sub-tropical and tropical regions and comprises about 200 species. The genus is found in the Americas, Africa (rarely), Asia and Australia (Sleumer, 1980; Mosaddik *et al.*, 2004; Breteler, 2008). In Brazil, there are about 48 species, 24 of which are endemic (Marquete & Vaz, 2007; Marquete & Mansano, 2010) and *Casearia arborea* (Rich.) Urb. is one of 12 species of the genus found in the State of Rio de Janeiro (Marquete & Vaz 2007; Marquete & Mansano, 2010).

Species of *Casearia* exhibit habits from subshrub to arboreal, with woody trunks and smooth to fissured bark, sometimes with globular or oblong lenticels on the branches. Leaves have translucent dots and dashes distributed on their surface and short and slender petioles. Inflorescences range from fasciculate to umbeliform with small bisexual flowers and greenish sepals. The flowers have 8-25 stamens with globular to oblong anthers arranged in fillets interspersed with disc lobes or in two whorls, superolateral ovary and produce tricarpellate fruit with persistent sepals (Marquete & Mansano, 2013). According to Chase *et al.* (2002), the taxonomic relationships in the Salicaceae family are still very controversial, making it a very heterogeneous group.

Thadeo *et al.* (2014) studied the anatomy of six plant species and found that there were anatomical characteristics inherent in *Casearia* genus such as secretory ducts and cavities, as well as crystal idioblasts. These idioblasts contain prismatic crystals of druses, which are often found throughout the leaf blade, including near the phloem. Moreover, cavities are present in the leaf blade and petiole, as well as two-armed and hook-shaped trichomes (Thadeo *et al.*, 2009; Thadeo *et al.*, 2014).

The secondary metabolism of *Casearia* is characterized by diterpenes, with special attention to clerodane-type (more than 100 diterpenes have already been isolated) (Kanokmedhakul *et al.*, 2007; Carvalho *et al.*, 2009; Ferreira *et al.*, 2010). Triterpenes and neolignans have been also described for *Casearia* (Raslan *et al.*, 2002; Wang *et al.*, 2010). The essential oils of *Casearia* genus are rich in non-oxygenated sesquiterpenes (Tininis *et al.*, 2006; Sousa *et al.*, 2007; Silva *et al.*, 2008a). Other *Casearia* species also present a predominance of sesquiterpenes, with β-caryophyllene as the major component for *C. grandiflora* Camb. and *C. decandra* Jacq. (Morais *et al.*, 2011; Stefanello *et al.*, 2010).

Notwithstanding, the essential oils of *Casearia* genus exhibited potent cytotoxic activities in tumor cell lines, including A549 cell line according to the study of Silva *et al.* 2008a. With respect to the work of Bou *et al.* (2013), sesquiterpenes were also identified in the essential oil of a species from the State of São Paulo, as far as it has been noted that cytotoxic activities are related to sesquiterpenes, although monoterpenes can be identified in the essential oil of *Casearia* genus as well.

In relation to *C. arborea*, Beutler *et al.* (2000) studied the phytochemistry from the roots of this species and identified clerodane diterpenes that exhibited cytotoxic potential in NCI-60 tumor cells. However, there is a complete absence of literature data related to the chemical profile of essential oils and their biological activities from *C. arborea* leaves. In this study, the secretory structures (oil-producing idioblasts) were identified in *C. arborea* leaves collected from trees growing in the city of Rio de Janeiro and the chemical profile of the essential oils and their cytotoxic effect on A549 tumor cell line were analysed.

MATERIALS AND METHODS

Study site and plant selection

Casearia arborea (Rich.) Urb. (Salicaceae) leaves were collected from mature trees growing in the Botanical Garden of Rio de Janeiro (S22°58’01.00” W43°14’48.30”), Brazil (authorization SISBIO number 24057-2). The identification was performed by Dr. Ronaldo Marquete and a voucher specimen was deposited in the Botanical Garden Herbarium of Rio de Janeiro with registration number RB 586916.

Histological analysis

Fully expanded leaves from the fourth node were collected from three individuals and fixed in 2.5% glutaraldehyde diluted in phosphate buffer 0.1 M, pH 7.2 (Karnovsky, 1965) followed by immersion in 70% alcohol. Leaf blade (midrib and intercostal region) as well proximal, medial and distal petiole samples were subsequently dehydrated in an ethanol series and in ethanol: propanone (2:1, 1:1 and 1:2 v/v) and propanone: ethanol solutions (2:1, 1:1, 1:2 v/v). After dehydration, the samples were included in historesin (hydroxyethyl methacrylate) according to Meira & Martins (2003). The materials were initially placed in a 100% alcohol: historesin solution (1:1 v/v) for a period of 8h. Then, three exchanges were carried out at intervals of 24 hours of pure historesin. A pure historesin: polymerizing solution (Hardener) 1: 0.066 (v/v) was prepared so that samples could be embedded in plastic molds. The samples were sectioned in Jung Heidelberg rotary microtome and the sections were stained with 0.05% toluidine blue (O'Brien *et al*., 1965) and mounted between slide and coverslip using Entellan. The images were captured in an Olympus BX53 optical microscope and Image-Pro Plus 7.0 digital camera at Department of Histology and Embryology (DHE), Biology Institute Roberto Alcantara Gomes (IBRAG), Rio de Janeiro State University (UERJ).

The epidermis analysis of fragments from the middle third of leaves fixed in 70% alcohol was performed in order to highlight the wall of epidermal cells, stomata type and presence of trichomes. The selected fragments were boiled in a 10% nitric acid solution (Ghouse & Yunus, 1972) until the epidermis dissociated. Subsequently, the epidermis was washed 3x in distilled water and placed in a 50% sodium hypochlorite solution for clarification. The samples were washed 3x in distilled water followed by 3x in a solution of distilled water and acetic acid 1:500 (v/v) and were mounted between the slide and cover slip in 50% glycerin medium. The number of stomata per mm^2 was counted in 25 fields. The description and classification of stomata were based on Wilkinson (1979).

The histochemical tests were performed with fresh leaves from the sixth node leaves. The tests were done as described in Table 1.

Table 1
Histochemical tests

Substances	*Test*	Reference	Method	*Wash*
Lipidic	Sudan III	Johansen (1940)	Sudan III (15 min)	EtOH 70% and water
	Sudan IV	Johansen (1940)	Sudan IV (15 min)	EtOh 70% and water
Lipidic, acid and neutral	Nile Blue sulphate test	Cain (1947)	Nile Blue sulphate test (60º C/5 min)	Acetic acid 1%, 60° C for 5 min and water
Total phenolic compounds	Ferric chloride	Johansen (1940)	Ferric chloride (25 min)	Water

Essential oil extraction and analysis

Fresh leaves of *C. arborea* (800 g) were cut into small pieces and submitted to hydrodistillation in a modified Clevenger apparatus for two hours. Essential oil was extracted from the aqueous phase, dried over anhydrous sodium sulfate, transferred to amber flasks and kept at -20º C until analysis and biological tests. The yield of essential oil was estimated to be 0.2%.

The essential oil sample was subjected to analysis by gas chromatography coupled to a flame ionization detector (HP-Agilent 6890 GC-FID) and by gas chromatography coupled to a mass spectrometer (HP Agilent GC 6890 – MS 5973), at the Analytical Platform of Institute of Pharmaceutical Technology, Farmanguinhos, Oswaldo Cruz Foundation, Rio de Janeiro as described by Oliveira *et al.* (2014). Briefly, the essential oil was diluted in dichloromethane (1000 µg/mL) and analyzed by GC-MS to obtain the mass spectra and the constituent chemicals were characterized. Concomitantly, another sample of essential oil (500 µg/mL) was analyzed by GC-FID for quantification of chemical constituents and to determine the retention indices (RI). Each essential oil component was quantified based only in the individual component's relative peak area in the chromatogram. The substances were identified by comparing their mass spectra with database registration (WILEY7n) and the calculated linear retention indices (RI) with records from literature (Adams, 2001). RI were calculated using GC data of a homologous series of saturated aliphatic hydrocarbons within C_8 to C_{20} (Sigma-Aldrich), performed using the same column and under the same conditions used in the GC analysis for the essential oils, using the equation proposed by Van

Den Dool and Kratz (1963).

GC-FID parameters

HP-5MS (5% diphenyl, 95% dimethylpolysiloxane) column (30 m × 0.32 mm i.d. × 0.25 μm particle size), temperature programming from 60 to 240° C, increasing at a rate of 3° C/min, using synthetic air and helium as the carrier gases, at a flow rate of 1000 μL/min and an injection volume of 1 μl.

GC-MS parameters

HP-5MS (5% diphenyl, 95% dimethylpolysiloxane) column (30 m × 0.32 mm i.d. × 0.25 μm particle size), temperature programming from 60 to 240° C, increasing at a of 3° C/min, using helium as the carrier gas, at a flow rate of 1000 μL/min and an injection volume of 1 μl.

Cell Culture

The A549 cell lineage was obtained from Microbiology Department from the Rio de Janeiro State University (Brasil). The cell lineage was maintained in continuous exponential growth by exchanging twice-a-week in a F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) containing 2 mM L-glutamine, 1500 mg/L sodium bicarbonate, 10% fetal bovine serum (Sigma-Aldrich Company, Saint Louis, MO,USA), 0.25 μg/mL glutamine (Sigma-Aldrich Company), 2.5 μg/mL amphotericin B (Sigma-Aldrich Company) and 5000 μg/mL gentamicin (Sigma-Aldrich Company). The cell lineage was kept in a humidified incubator containing 5% CO_2 in air at 37° C and split regularly before attaining 70–80% confluence.

Vero cells (African green monkey kidney) were grown in Eagle's minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 50000 μg/mL gentamicin, 2500 μg/mL fungizone and 10% heat-inactivated fetal bovine serum (FBS), and maintained at 37° C in 5% CO_2 atmosphere.

Cytotoxic assay

According to the WST-1 assay, the mitochondrial dehydrogenase (succinatetrazolium-reductase) activity was determined by colorimetric assay (Roche Diagnostics, Meylan, France). Formazan dye (10 ml) was added to each well prior to 20 minutes incubation at 37ºC. Absorbance was measured in triplicate at 450 nm with a multi-well spectrophotometer (Celer – Polaris). Concentrations ranging from 0.5 to 20 μg/mL of the *C. arborea* essential oil, diluted in 0.1% dimethyl sulfoxide (DMSO) were used. A commercial drug doxorubicin (DOXO), commonly used in chemotherapy (Ghasemi *et al*., 2016), was tested as positive control at concentrations of 0.01358 μg/mL (EC_{50}). Negative controls consisted of DMSO 0.1% in saline. The results were expressed as a percentage of cell viability relative to the control.

Statistics

Data are reported as the mean ± SD for at least three replicates. Statistical analyses were performed using a Student-t test, with the significance level set at $p < 0.05$.

RESULTS

Epidermis

In the intercostal region, epidermal cells on the adaxial surface, frontally viewed, have straight periclinal walls with sinuous contours (Figure 1A). Stomata are present only in the abaxial surface (hypostomatic) at a frequency of 62.2 stomata per mm^2. Stomata were observed in anomocytic and anisocytic standards (Figure 1B).

Trichomes

Uniseriate glandular trichomes were observed in the intercostal region, occurring in the abaxial surface, especially along the ribs (Figure 1C). Trichomes occur alone, in hook form (Figure 1D and 1E). Cork-warts are randomly arranged across the leaf (Figure 1 F).

Mesophyll

The mesophyll consists of palisade parenchyma with irregularly shaped cells, which were not arranged into organized rows. This parenchyma is discontinuous in certain areas with disruptions occurring at the vascular bundles and locations of cavities (Figure 2A). The spongy parenchyma may have four to five layers and cells in this parenchyma have an irregular shape and are sparsely arranged. Idioblasts containing druse crystals are present.

Leaf margin

The leaf margin in cross section has epidermal cells with a tabular shape on the adaxial surface and a more rounded and irregular shape on the abaxial surface. Both the palisade and the spongy parenchyma are diffused and without boundaries to differentiate them. It is noteworthy cavities were also observed in this region (Figure 2B).

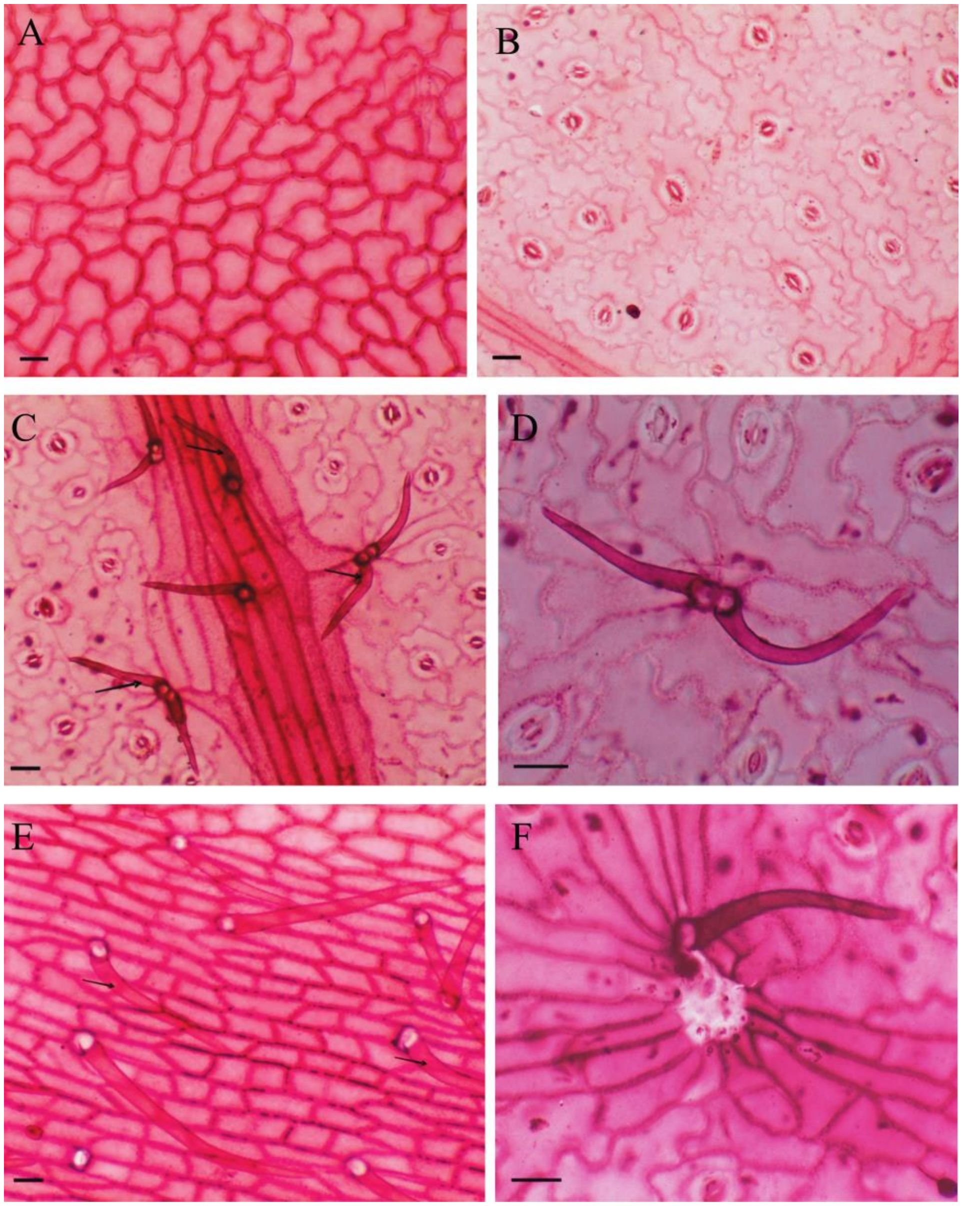

Figure 1
A: adaxial surface with straight periclinical and undulate walls. B: abaxial surface showing stomata of anomocytic and anisocytic standards. C: detail of solitaire and two-armed (→)tector trichomes along the ribs. D: detail of two-armed tector trichome. E: solitary tector trichomes on midrib of the adaxial surface. F: uniseriate tector trichome and cork-wart structure. Bars: A, B, C, E: 20 µm; D, F: 40 µm

Secretory cavities

The secretory cavities are located in the petiole, in the spongy parenchyma, close to the vascular bundles and also near the phloem (Figure 2C). Prismatic crystals were also observed in the parenchyma (Figure 2D). Cavities are distributed throughout the lamina, both in the mesophyll and close to the epidermis. Secretory cavities consist of wide intercellular spaces surrounded by epidermal cells which secrete chemicals into the cavities (Figure 3A). The histochemical tests Sudan III, Sudan IV and Nile Blue confirmed the presence of lipophilic contents in the cavities, with lipid droplets inside (Figure 3B, 3C and 3D). 12 and 14). Idioblasts with phenolic compounds were also identified (Figure 3E and 3F).

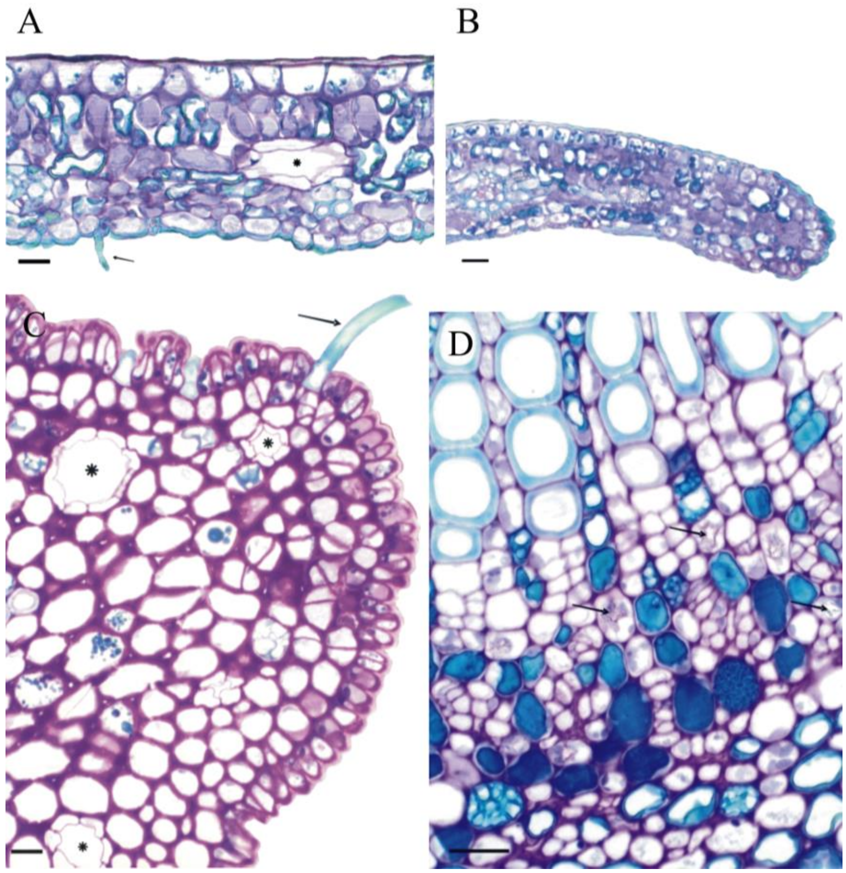

Figure 2
A: mesophyll consists of palisade parenchyma with irregular shaped cells and exhibits cavity (*) and tector trichome (→) on abaxial surface.
B: leaf margin shows palisade and spongy parenchymas difused and without boundaries to differentiate them.
C: detail of lateral projection on adaxial surface with cavities (*) and uniseriate tector trichome (→).
D: detail of druses (→) and idioblasts with phenolic compounds. Bars: A, C: 20 μm; B: 10 μm; D: 40 μm

Chemical analysis of the essential oil by GC-MS and GC-FID allowed the characterization of 37 compounds, comprising 97.9% of the essential oil from the leaves of *C. arborea* presented in Table 2. All compounds were identified as sesquiterpenes. Monoterpenes were not identified. The essential oil exhibited cytotoxic activity against A549 tumor cells with EC_{50} at 4.0 μg/mL and has dose dependent pattern (r = -0.79, p = 0.03) as determined by linear regression test. On the other hand, cytotoxic effects were not observed in Vero cell line (Table 3).

DISCUSSION

Anatomical aspects

With respect to leaf chemistry, cavities reacted positively to Nile Blue sulfate, Sudan III and IV, confirming the lipophilic nature of their contents (Figure 3B, 3C and 3D). The lipophilic content of these structures may be characterized by the presence of volatile components such as essential oils. Essential oils are part of the secondary metabolism of plants, and may be associated with defense, given that substances present in both essential oils and extracts have antimicrobial activity (Da Silva *et al.*, 2006; Silva *et al.*, 2008b).

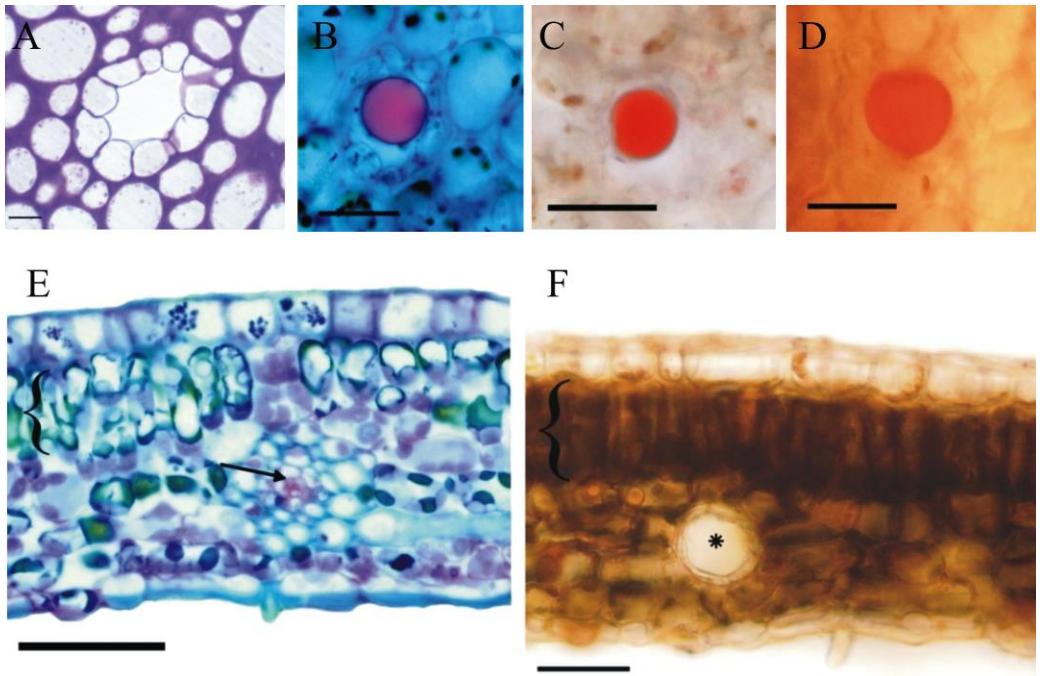

Figure 3
A: detail of cavity on midrib surrounded by epidermal cells
B: cavity with lipophilic contents which reacted positively to Nile Blue sulfate
C: cavity with lipophilic contents which reacted positively to Sudan IV
D: cavity with lipophilic contents which reacted positively to Sudan III
E: detail of palisade parenchyma with phenolic idioplasts ({) and vascular bundle (→)
F: palisade parenchyma with phenolic idioblasts which reacted positively to ferric chloride on palisade parenchyma ({) and detail of cavity (*).
Bars: A, B, C, D, E, F: 40 μm

Chemical analysis of the essential oil

In accordance with recent studies, monoterpenes (such as α-pinene and linalool) may occur in the essential oils of some species of *Casearia* (Sousa *et al.*, 2007; Silva *et al.*, 2008a; Stefanello *et al.*, 2010) but they were not found in the sample. An interesting characteristic of the volatile fraction was the high proportion of non-oxygenated sesquiterpenes (90.2%), in which byciclogermacrene (18.7%), germacrene D (12.1%) and α-humulene (11.5%) were identified. Oxygenated sesquiterpenes comprised 7.7% of the sample and consisted mainly of *epi*-α-cadinol. The main compounds identified in the sample are very common in the essential oil from leaves of *C. sylvestris* (Tininis *et al.*, 2006; Silva *et al.*, 2008a; Esteves *et al.*, 2008; Bou *et al.*, 2013); a species that also occurs in the State of Rio de Janeiro. The sesquiterpenes byciclogermacrene, germacrene D and α-humulene are structurally related hydrocarbons, since their biosynthesis is from the acetate-mevalonate pathway, via the precursor *E*,*E*-farnesyl pyrophosphate.

Cytotoxic activity

Essential oil of *C. arborea* applied at concentrations ranging from 0.5 to 20 μg/mL significantly reduced the proliferation of A549 compared with the control (culture medium with FBS). The essential oil showed cytotoxic activity against A549 tumor cells with an EC_{50} of 4.0 μg/mL and had a dose dependent pattern (r = -0.79, p = 0.03) as determined by a linear regression test are exhibited in Table 3. The effect of DOXO, the commercially anti-cancer drug, was also exhibited and had an EC_{50} of 0.01358 μg/mL. The solvent used (DMSO 0.1%) to re-suspend the essential oil of *C. arborea* had no effect on the normal proliferation (data not shown).

Table 2
Chemical composition of the essential oil from fresh leaves of *C. arborea*

Compounds	RI_{calc}	RI_{lit}	Percentage (%)
Non-Oxygenated Sesquiterpenes		n = 24	90.2
δ-Elemene	1336	1339	3.3
α-Cubebene	1349	1351	3.7
Isoledene	1370	1373	0.4
α-Copaene	1379	1376	4.8
Daucene	1385	1380	0.3
Isolongifolene	1390	1387	1.1
β-Elemene	1393	1391	2.5
α-Gurjunene	1409	1409	1.3
(*E*)-Caryophyllene	1418	1414	6.2
γ-Elemene	1434	1433	4.4
γ-guaiene	1440	1439	1.1
α-*neo*-Clovene	1454	1454	0.6
α-Humulene	1458	1454	11.5
allo-Aromadendrene	1464	1461	1.7
γ-Muurolene	1476	1477	0.3
Germacrene D	1485	1480	12.1
Valencene	1496	1490	1.2
Byciclogermacrene	1498	1494	18.7
trans-β-Guaiene	1501	1500	0.3
γ-Cadinene	1510	1513	2.3
δ-Cadinene	1520	1524	7.4
Cadina-1,4-diene	1530	1532	0.2
α-Cadinene	1534	1538	0.2
Germacrene B	1555	1556	4.6
Oxygenated Sesquiterpenes		n = 13	7.7
Ledol	1564	1565	0.3
Germacrene D-4-ol	1575	1574	0.6
Caryophyllene oxide	1577	1581	0.1
Globulol	1583	1583	0.9
Viridiflorol	1590	1590	0.6
Carotol	1595	1594	0.5
Humulene epoxide II	1602	1606	0.3
α-Acorenol	1625	1630	0.3
β-Acorenol	1630	1634	0.2
epi-α-Cadinol	1638	1640	2.0
α-Muurolol	1642	1645	0.2
α-Cadinol	1650	1653	1.5
NI	1662	1666	0.2
Total of Identified Compounds %		n = 37	97.9

References: RI_{cal}: Retention Index values calculated, RI_{lit}: Retention index values from literature data, n: number of identified compounds, NI: Not identified compounds.

The observed cyctoxicity of the essential oil from *C. arborea* leaves is consistent with results from other studies of the cytotoxic activity of essential oils from Salicaceae in tumor cell lines (Nikolic *et al.*, 2014; Hayan *et al.*, 2016). For instance, essential oil of *C. sylvestris* showed potent cytotoxic activity against three tumor cell lines (A549, He-La and HT-29, EC_{50} 63.3, 60.7 and 90.6 μg/mL, respectively), with the major components identified as β-caryophyllene and α-humulene (Silva *et al.*, 2008a). Similarly, Bou *et al.* (2013) reported that essential oil from *C. sylvestris* collected in São Paulo showed cytotoxic effects against eight tumor cell lines (B16F10, B16F10-nex12, A2058, U87, HL-60, Siha, MCF-7, HeLa), with major component of this essential oil being α-zingiberene.

However, there are no previous studies related to the chemical profile of the essential oil from leaves of *C. arborea* or its cytotoxic activity. Here we demonstrate for the first time that the essential oil of *C. arborea* showed cytotoxic effects on tumor cell line A549 (EC_{50} at 4.0 μg/mL) with the major component being bicyclogermacrene. These results together with those from other studies indicate that sesquiterpenes have widespread cytotoxic activity. Therefore, further chemical and biological studies of *C. arborea* and other *Casearia* species are warranted.

Table 3
Cytotoxic effect of the essential oil from fresh leaves of *C. arborea* in A549 cell line

Samples	MNTC μg/ml	CC_{50} μg/ml (Vero cell)	EC_{50} μg/ml (A549)	CC_{50} μg/ml (A549)
Essential oil	≥250 (Vero cell)	>250	4.0	20.0
Doxorrubicina	0.05420 (A549)	-	0.01358	0.02168

References: MNTC: maximum non-toxic concentration, CC_{50}: 50% cytotoxic concentration, EC_{50}: effective concentration, A549: human lung carcinoma.

CONCLUSION

Casearia arborea leaf anatomy and the chemical profile and cytotoxic activity of the essential oil were described for the first time. The leaves were found to contain secretory cavities characterized by a wide intercellular space surrounded by epidermal cells which secreted compounds including lipophilic compounds such as essential oil. The essential oil was composed mainly of non-oxygenated sesquiterpenes and showed strong cytotoxic activity against A549 tumour cells. This research contributes to the knowledge regarding the essential oil in *C. arborea* of this species including: leaf storage, its chemical profile and its biological properties.

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