© 2017
Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 16 (5): 506 - 512
ISSN 0717 7917
www.blacpma.usach.cl

Artículo Original | Original Article

Cytotoxic effects of the essential oil from leaves of *Casearia sylvestris* Sw. (Salicaceae) and its nanoemulsion on A549 tumor cell line

[Efectos citotóxicos del aceite esencial de las hojas de *Casearia sylvestris* Sw. (Salicaceae) y su nanoemulsión sobre líneas celulares tumorales A549]

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Abstract: Extracts from leaves of *C. sylvestris* have cytotoxic effect in different tumor cell lines, possibly due to clerodane type diterpenes (casearins). On the other hand, there are few studies related to the antitumor activity of the essential oils from this species. This work evaluated for the first time the cytotoxicity effects of the pure essential oil and its nanoemulsion against A549 tumor cell line (human lung carcinoma). The essential oil was obtained from fresh leaves by hydrodistillation in a Clevenger-type apparatus and analyzed by GC/MS and GC/FID. Cytotoxicity evaluation was performed using the WST-1 test. The chemical analysis of the essential oil revealed a volatile fraction composed mainly of non-oxygenated sesquiterpenes (72.1%). The essential oil and its nanoemulsion exhibited cytotoxic activity against A549 tumor cells with EC_{50} of 4.0 µg/mL and EC_{50} of 1.0 µg/mL, respectively. Both samples displayed a dose dependent pattern ($r = -0.79$, $p = 0.03$) as determined by linear regression test.

Keywords: α-humulene; A549; *Casearia* genus; essential oil; nanosystem; sesquiterpenes.

Resumen: los extractos de las hojas de *Casearia sylvestris* tienen efectos citotóxicos en diferentes líneas celulares tumorales, posiblemente debido a los diterpenos tipo clerodane (casearinas). Por otra parte, hay muy pocos estudios relacionados con la actividad antitumoral del aceite esencial de estas especies. Este trabajo evalúa por primera vez el efecto citotóxico del aceite esencial puro y su nanoemulsión contra la línea de células tumorales A549 (carcinoma humano de pulmón). El aceite esencial fue obtenido de hojas frescas por hidrodestilación en un aparato tipo Clevenger y analizado por GC/MS y GC/FID. La evaluación de citotoxicidad fue realizada usando la prueba WST-1. El análisis químico del aceite esencial reveló una fracción volátil compuesta principalmente por sesquiterpenos no oxigenados (72,1%). El aceite esencial y su nanoemulsiónexhibió actividad citotóxica contra las células tumorales A549 con una EC_{50} de 4,0 µg/mL y una EC_{50} de 1,0 µg/mL, respectivamente. Ambas muestras exhibieron un patrón dosis-dependiente ($r = -0,79$, $p = 0,03$) determinado por análisis de regresión lineal.

Palabras clave: α-humuleno; A549; aceite esencial; género *Casearia*; nanosistema; sesquiterpenos.

Recibido | Received: March 7, 2017
Aceptado | Accepted: April 7, 2017
Aceptado en versión corregida | Accepted in revised form: April 8, 2017
Publicado en línea | Published online: September 30, 2017
Declaración de intereses | Declaration of interests: The authors are extremely grateful to Brazilian agencies CNPq and Faperj.
Este artículo puede ser citado como / This article must be cited as: FG Pereira, R Marquete, LO Cruz, DQ Falcão, E Mansur, DL Moreira. 2017. Cytotoxic effects of the essential oil from leaves of *Casearia sylvestris* Sw. (Salicaceae) and its nanoemulsion on A549 tumor cell line. **Bol Latinoam Caribe Plant Med Aromat** 16 (5): 506 – 512.

INTRODUCTION

Casearia sylvestris Sw. belongs to Salicaceae family, and can be found throughout Brazilian territory (Marquete & Mansano, 2010) and it is one of the 71 plants of interest of the Brazilian public health system (SUS), since is widely used as antimicrobial, anti-inflammatory, anti-herpes and antitumor (Esteves *et al.,* 2005; Dos Santos *et al.,* 2010; Bratti *et al.,* 2013; Felipe *et al.,* 2014). Recently we have described the anti-herpes and antitumor activities of the essential oil from leaves of Casearia species (Pereira *et al.,* 2016; Pereira *et al.,* 2017).

The secondary metabolites of *Casearia* are based on diterpenes, with special attention to clerodane type (over one hundred diterpenes have been isolated) (Carvalho *et al.,* 2009; Ferreira *et al.,* 2010). Triterpenes, lignans, neolignans, galic acid derivatives and flavonoids have been also described for *Casearia* extracts (Raslan *et al.,* 2002; Wang *et al.,* 2010; Ferreira *et al.,* 2014; Felipe *et al.,* 2014). Similar results of leaf extract demonstrated previously antitumor action of a fraction rich with casearins (clerodane diterpenes) and its main component (Casearin X) that was isolated from *C. sylvestris* (Ferreira *et al.,* 2016).

The essential oils of *Casearia* genus are rich in sesquiterpenes (Esteves *et al.,* 2005; Tininis *et al.,* 2006; Sousa *et al.,* 2007; Silva *et al.,* 2008). For instance, the sesquiterpene α-zingiberene was the main compound found in the essential oil of plants collected in São Paulo city, and exhibited cytotoxic activity against tumor cell lines (Bou *et al.,* 2013). In addition there are also studies demonstrating the cytotoxic effect of essential oils rich in sesquiterpenes from different plants families, especially α-caryophyllene and β-caryophyllene (Sylvestre *et al.,* 2005; Sylvestre *et al.,* 2006).

Although there are few studies on the cytotoxic effect on different tumor cell lines for the essential oil of C. sylvestris collected from different sites (Silva *et al.,* 2008; Bou *et al.,* 2013), it is the development of nanoemulsions from essential oils to improve stability and activity is highly desirable (Li *et al.,* 2016). In this work we investigated, for the first time, the action of the pure essential oil of *C. sylvestris* collected in Rio de Janeiro (Tijuca National Park site) and its nanoemulsion on A549 tumor cell line. Since this species is widely used as medicinal plant by the Brazilian population, it is very important to confirm these activities and to develop a possible therapeutic delivery nanosystem of water insoluble compounds.

MATERIALS AND METHODS

Study site and plant selection

Casearia sylvestris Sw. (Salicaceae) was collected in Tijuca National Park (S22°57'05.04" W43°17'10.09"), Rio de Janeiro, Brazil (SISBIO license n. 38765-1 /CGEN license n. 010105/2014-0). Plant identification was performed by Dr. Ronaldo Marquete, and the herbarium voucher was deposited in the Botanical Garden Herbarium of Rio de Janeiro with registration number RB 570651.

Essential oil extraction and analysis

Fresh leaves of *C. sylvestris* (1.5 kg) were chopped into to small pieces and led to hydrodistillation in a modified Clevenger-type apparatus for two hours. Essential oil was directly separated from the aqueous phase yielding 1.2% (v/w), transferred to amber flasks and kept at low temperature (-20º C) until analysis. The sample was subjected to analysis by gas chromatography coupled to flame ionization detector (HP-Agilent 6890 GC/FID) and by gas chromatography coupled to mass spectrometry (HP Agilent GC 6890 - MS 5973). Identification of the compounds was done according to comparison of the mass fragmentation pattern and retention indices as described before (Pereira *et al.,* 2017).

GC/FID analysis 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, with increase of 3° C/min, using helium as the carrier gases, with a flow rate of 1 mL/min and injection volume of 1 μL.

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Nanoemulsion preparation

Nanoemulsion preparation was performed as described by Pereira *et al.* (2016). Briefly, the nanoemulsion was prepared with a final volume of 25 mL, containing 5% pure essential oil, 5% surfactant and 90% water. The material was stored at room temperature (20 ± 2° C) and the droplet size distribution analysis was evaluated 7 days after preparation.

Droplet size analysis

Mean droplet size and polydispersity (PDI) of the nanoemulsion were determined by Dynamic Light Scattering (DLS) (Zetasizer ZS90, Malvern, UK).

Cell Culture

The A549 cell lineage was obtained from Microbiology Department from the Rio de Janeiro State University (Brasil). Cells were maintained in continuous exponential growth by twice-a-week exchanging in a F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) containing 2 mM L-glutamine, 1500 mg/L of sodium bicarbonate, 10% of fetal bovine serum (Sigma-Aldrich Company, Saint Louis, MO, USA), 0.00025 mg/mL of glutamine (Sigma-Aldrich Company), 0.0025 mg/mL of amphotericin B (Sigma-Aldrich Company) and 5mg/mL of 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 (Dantas *et al.*, 1996).

Cytotoxic assay

The mitochondrial dehydrogenase (succinatetrazolium-reductase) activity was determined by colorimetric assay (Roche Diagnostics, Meylan, France), according to the WST-1 test. Formazan dye (10 mL) was added to each well prior to 20 minutes incubation at 37º C. Absorbance was measured in triplicate at 450 nm with a multi-well spectrophotometer (Celer – Polaris). Different concentrations of the *C. sylvestris* essential oil diluted in 0.1% of dimethyl sulfoxide (DMSO), ranging from 0.5 to 20 µg/mL were used. A commercial drug doxorubicin (DOXO), commonly used in chemotherapy was tested as positive control. Negative controls were done with DMSO 0.1% in saline. The results were expressed in percentage of cell viability in comparison to the control.

Statistical

Statistical analysis was performed using the ANOVA and Tukey–Kramer multiple comparison tests by the statistical program InStat 3.01 version (GraphPad Software, San Diego, CA, USA). The significance level of $p < 0.05$ was taken as statistical significance, and used to compare data within the same experiment.

RESULTS

According to GC/MS, GC/FID and Kovats Indices analysis, it was possible to characterize 21 compounds, all sesquiterpenes, comprising 98.2% of the essential oil from leaves of *C. sylvestris* (Pereira *et al.*, 2016). The main compounds identified were α-humulene (17.8 %), spathulenol (11.8%), and α-copaene (8.5%). Monoterpenes and arylpropanoids, common compounds identified in essential oils from higher plants, were not found. The stable nanoemulsion for delivering the essential oil components displayed an average size of 212.9 ± 4.0 nm (PDI = 0.213 ± 0.035).

Essential oil of *C. sylvestris* ranging from 0.5 to 10 µg/mL reduced significantly the A549 proliferation as compared to the control (culture medium with FBS), showing an EC_{50} of 4.0 µg/mL, and a dose dependent pattern ($r = -0.79$, $p = 0.03$) as determined by linear regression test. While the cytotoxic concentration (CC_{50}) corresponded to 10 µg/mL in A549 cells, toxicity in non-tumor Vero cells was only observed at concentrations above 250 µg/mL, representing a great selectivity index (> 62.5). The nanoemulsion was more active, showing an EC_{50} of 1.0 µg/mL.

DISCUSSION

Chemical analysis of the essential oil

Essential oils comprise a mixture of secondary metabolites of plants, mainly composed by monoterpenes, sesquiterpenes and arylpropanoids (Santos *et al.*, 2001). Studies performed with the essential oils of *C. sylvestris* collected at different sites demonstrated that these oils are rich in sesquiterpenes, but the major compounds are variable according to the site of collection (Silva *et al.*, 2008) (Table 1). It was observed that there was no change in the chemical composition of the major components (germacrene D and germacrene B) from the essential oil of individuals of *C. sylvestris* collected in the State of São Paulo (Tininis *et al.*, 2006). The study of species collected in Minas Gerais State (Esteves *et al.*, 2005) showed that the major components of the essential oil were bicyclogermacrene (40.9%) and β-acoradiene (20.8%), while the components of plants collected in Santa Catarina exhibited predominance of sesquiterpenes (86.8%) with major compounds identified as β-caryophyllene (27.5%) and bicyclogermacrene (24.2%). However, the monoterpene α-pinene could be also identified in this

Table 1
Chemical compounds of the essential oil from leaves of *C. sylvestris*

Compounds	RI_{calc}	RI_{lit}	Percentage (%)
Non-oxygenated Sesquiterpenes	***n = 14***		***72.1***
α-Cubebene	1354	1351	7.2
α-Copaene	1382	1376	8.5
β-Cubebene	1394	1390	1.7
β-Elemene	1396	1391	3.8
(*E*)-Caryophyllene	1414	1418	7.6
γ-Elemene	1426	1433	4.8
α-Humulene	1451	1454	17.8
Seichellene	1455	1460	2.4
γ-Muurolene	1474	1477	0.1
Germacrene D	1476	1480	3.1
Byciclogermacrene	1491	1494	3.1
γ-Cadinene	1508	1513	2.5
7-*epi*-α-Selinene	1513	1517	2.1
Germacrene B	1555	1556	7.4
Oxygenated Sesquiterpenes	***n = 7***		***25.6***
Sphatulenol	1570	1576	11.8
Caryophyllene oxide	1575	1581	3.5
Humulene epoxide II	1600	1606	4.1
1-*epi*-Cubenol	1620	1627	1.8
γ-Eudesmol	1629	1630	2.6
14-Hydroxi-9-*epi*-β-caryophyllene	1664	1663	0.5
α-Bisabolol	1681	1683	1.8
Total of identified compounds n, %	***n = 21***		**98.2**

References: RI_{cal}: Retention Index values calculated, RI_{lit}: Retention index values from literature data.

essential oil, but in small amount (Sousa *et al.*, 2007). In our work, the main compounds found in the essential oil of *C. sylvestris* collected in Rio de Janeiro State were also non-oxygenated sesquiterpenes, confirming studies previously published. However the major compound was identified as α-humulene. Again, our data reinforce that there is a chemical difference in the essential oils composition in accordance with the site of collection, which can result in different biological activities.

In the present work, the developed nanoemulsion increased stability, allowed dispersing non-polar compounds in aqueous phase (Pereira *et al.*, 2016) and significantly increased cytotoxic activity ($p < 0.05$)(Table 2). The development of nanosystems from natural products has promoted several researches on the pharmacological level. According to recent studies, essential oils or extracts can be incorporated into nanocarriers systems for better effectiveness of their active compounds (Ostertag *et al.*, 2012). However, there are few studies on the development of nanosystems with essential oils, especially for medicinal species occurring in Brazil (Duarte *et al.*, 2015). Thus, the development of nanosystems is a promising field of research for native species, which may possibly lead to pharmaceutical formulation for biologically active molecules for the treatment of different diseases, including cancer.

Cytotoxic activity

According to a recent study, wherein the essential oil

of *C. sylvestris* exhibited potent cytotoxic effect on tumor cell lines (B16F10, B16F10-nex12, A2058, U87, HL-60, Siha, MCF-7, HeLa), the main components were identified as α-zingiberene (48.31%) and *E*-caryophyllene (14.27%) (Bou *et al.*, 2013). In addition, other study showed that the main components of the essential oil of this species were bicyclogermacrene (43.6%), β-caryophyllene (18.1%) and spathulenol (15.9%), and its essential oil also exhibited cytotoxic activities on tumor cell lines A549, He-La and HT-29 (EC_{50} 63.3, 60.7 and 90.6 μg/mL, respectively) (Silva *et al.*, 2008). Here, demonstrated that the essential oil from leaves of C. sylvestris, and its nanoemulsion showed a much higher cytotoxic effect on tumor cell line A549 (EC_{50} at 4.0 μg/mL), and that the major component was α-humulene (17.8 %). These data confirm that sesquiterpenes have cytotoxic activity, and indicating that further chemical and biological studies with other *Casearia* species are extremely relevant (Stefanello *et al.*, 2010).

Table 2
Cytotoxicity and antitumor activities of the essential oil and nanoemulsion from fresh leaves of *C. sylvestris*

Sample	MNTC μg/mL (Vero cell)	CC_{50} μg/mL (Vero cell)	EC_{50} μg/mL (A549)
Essential oil	≥250	>250	4.0
Nanoemulsion	0.004	0.012	1.0
Doxorrubicina	0.05420	-	0.01358

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

CONCLUSION

Casearia sylvestris essential oil and its nanoemulsion showed strong cytotoxic activity against A549 tumour cells and other studies can be conducted for the contribution of chemical profiles and biological properties from species of Atlantic Forest.

ACKNOWLEDGEMENTS

The authors are extremely grateful to Brazilian agencies CNPq and Faperj.

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