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DNA damages promoted by the essential oil from leaves of *Casearia sylvestris* Sw. (Salicaceae)

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The *Casearia* species (Salicaceae) occur in the tropics and subtropics and their extracts are rich in clerodane-type diterpenes, known as casearins. According to the literature, extracts from *Casearia sylvestris* exhibit cytotoxic and genotoxic effects in different tumor cell lines, possibly related to the casearins. On the other hand, there are few studies related to the DNA damages of the essential oils from this species. This study is aimed at evaluating DNA damages promoted by the essential oil from leaves of *C. sylvestris* collected in Rio de Janeiro. The essential oil was obtained from fresh leaves (1.5 kg) by hydrodistillation for 2 h in a Clevenger-type apparatus, and analyzed both by gas chromatography coupled to a mass spectrometer (GC-MS) and gas chromatography coupled to a flame ionization detector (GC-FID). These analyses revealed a very diversified (*n* = 21 compounds) volatile fraction composed mainly of non-oxygenated sesquiterpenes (72.1%), and the major component was identified as α-humulene (17.8%). Genotoxicity was evaluated by the comet assay, showing DNA damages, mainly of classes 3 and 4 at 4.0 µg/ml (*p* < 0.05) according to the damage index (DI). This is the first demonstration of DNA damages in response to the essential oil of *C. sylvestris.*

Key words: A549, α-humulene, comet assay, sesquiterpenes.

INTRODUCTION

Casearia sylvestris Sw. (Salicaceae) can be found throughout the Brazilian territory, including the State of Rio de Janeiro (Marquete and Vaz, 2007; Marquete and Mansano, 2013), and is popularly known as "guaçatonga". This species presents several medicinal properties, according to ethnobotanical surveys (Tomazi

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et al., 2014). It is one of the 71 plants of interest of the Brazilian Public Health System (Sistema Único de Saúde (SUS)), due to its wide use by the population to treat different conditions, such as herpes and tumors (Pereira et al., 2016; Ferreira et al., 2016).

Recent studies reported that the essential oils of *Casearia* genus are rich in sesquiterpenes, while monoterpenes are rare and arylpropanoids are absent (Esteves et al., 2005; Tininis et al., 2006; Sousa et al., 2007; Silva et al., 2008). In addition, the sesquiterpenes showed cytotoxic activity against different tumor cell lines (Silva et al., 2008; Bou et al., 2013) as well as genotoxic effects (Péres et al., 2009; Maistro et al., 2010; Ortiz et al., 2016). However, although this species is widely used by the Brazilian population, possible genotoxic effects promoted by the essential oil from leaves have not been investigated so far.

MATERIALS AND METHODS

Plant collection

C. 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 collected in June 2014, chopped into to small pieces and led to hydrodistillation in a modified Clevenger-type apparatus for 2 h. Essential oil was directly separated from the aqueous phase yielding 1.2% (w/v), transferred to amber flasks and kept at low temperature (-20°C) until analysis. Essential oil 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), at the Analytical Platform of Institute of Pharmaceutical Technology, Farmanguinhos, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Briefly, the essential oil was diluted in dichloromethane (1.0 mg/ml) and analyzed by GC-MS to obtain the mass spectra and to perform chemical characterization. Concomitantly, another sample of essential oil (0.5 mg/ml) was analyzed by GC-FID for quantification of chemical constituents and to determine the retention indices (RI). The relative abundance of each essential oil component in the sample was quantified based in the individual component's relative peak area in the chromatogram. The substances in the essential oil were identified by comparing their mass spectra with database registration (WILEY7n) and by comparison of calculated Retention Indices (RI) with those from the literature (Adams, 2001; Pereira et al., 2016). RI were calculated from GC data of a homologous series of saturated aliphatic hydrocarbons within C8 to C20 (Sigma-Aldrich), performed using the same column and conditions used in the GC analysis for the essential oils, and with the equation proposed by Van dendool and Kratz (1963).

Evaluation of cell viability by the clonogenic assay

The A549 cell lineage was obtained from Microbiology Department from the Rio de Janeiro State University (Brazil). The cytotoxic potential of the essential oil was measured by the mitogenic capacity of treated cells using the clonogenic assay (Franken et al., 2006).

Comet assay

The procedure of comet assay was performed as described by Tice (2000), Cestari et al. (2004), and Miyaji et al. (2004) with modifications. Briefly, for each slide, 50 nuclei were randomly observed, always from the left to the right side, and total scoring (TS) was by obtained multiplying the number of cells in each class (nx) by the damage class (TS = (0 × n0) + (1 × n1) + (2 × n2) + (3 × n3) + (4 × n4)), ranging from 0 to 150 (Dantas et al., 2002). Nuclei were visually classified according to the migration of fragments (tail size) to one of five classes as follows: class 0 (undamaged nuclei, absence of tail), class 1 (nuclei with short tails, smaller than their diameter), class 2 (tail length one to two times the diameter of the nuclei), class 3 (tails twice or the diameter of the nuclei), and class 4 (nuclei maximally damaged).

Statistical analysis

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 adopted to compare data within the same experiment.

RESULTS

Essential oil chemical profile

According to GC-MS, GC-FID and Retention Indices (RI) analysis, it was possible to characterize 21 compounds, comprising 98.2% of the essential oil from leaves of *C. sylvestris* (Table 1).

Cytotoxic and genotoxic activities

The essential oil exhibited cytotoxic activity against A549 tumor cells, with EC_{50} of 4.0 µg/ml, and a dose dependent pattern ($r = -0.79$, $p = 0.03$), as determined by linear regression test. It is interesting to note that no cytotoxic effect was detected on Vero cells (African green monkey kidney, maximum nontoxic concentration ≥ 250 µg/ml) (Table 2). The essential oil also presented suppressive effects on the colony-forming ability on A549 cells after the treatment. The results of the comet assay are shown in Table 3. All types of damages could be observed, especially classes 3 and 4 ($p < 0.05$), when compared with the control.

DISCUSSION

The comet assay was performed to evaluate potential genotoxic effects of essential oils, considering that it is a

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

Components	RI_{calc}	RI_{lit}	%
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-Elemeno	1426	1433	4.8
α-Humulene (α-caryophyllene)	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-Hydroxy-9-*epi*-β-caryophyllene	1664	1663	0.5
α-Bisabolol	1681	1683	1.8
Total of identified compounds n, %	**n = 21**		**98.2**

RI_{cal} = Retention Index values calculated;RI_{lit} = Retention index values from literature data.

Table 2. Cytotoxicity activity of the essential oil from fresh leaves of *C. sylvestris* on 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	>250	4.0	10.0
Doxorrubicina	0.05420	-	0.01358	0.02168

MNTC: Maximum non-toxic concentration; CC_{50}: 50% cytotoxic concentration; EC_{50}: effective concentration; A549: human lung carcinoma.

Table 3. Comet assay scores for the essential oil of *C. sylvestris* on A549 tumor cell line.

Samples	Number of cells		Classes				
	Analyzed	Tailed	0	1	2	3	4
Control	100	40	2	58	20	15	5
Essential oil (4 µg/ml)	100	79	6	15*	22	30*	27*

Class 0: Undamaged nucleus, no tail; Class 1: nucleus with a short tail; Class 2: tail length is 1 and 2 times the diameter of the head; Class 3: tail is longer than the diameter of the nucleus; Class 4: nucleus totallydamaged; *p<0.05.

rapid and sensitive method to measure DNA damages (Tice et al., 2000; Almeida et al., 2012). Evaluation of cytotoxicity by clonogenic assay, and DNA damage of the essential oil have also been investigated. Considering

that *C. sylvestris* is widely used in folk medicine, it is important to verify the toxic effects of its volatile fractions, especially considering that the National Sanitary Surveillance Agency (Agência Nacional de Vigilância Sanitária - ANVISA) recommends the assessment of possible mutagenicity of medicinal plants (Balbino and Dias, 2010). *C. sylvestris* essential oil is composed exclusively by sesquiterpenes and the main identified compounds were α-humulene (17.8%), spathulenol (11.8%), and α-copaene (8.5%) (Pereira et al., 2016). For instance, sesquiterpenes were also identified as the major compounds in the essential oils from the leaves of *C. sylvestris* collected in the Brazilian states of São Paulo, Santa Catarina and Minas Gerais (Tininis et al., 2006; Silva et al., 2008; Esteves et al., 2008; Bou et al., 2013). However, the analyzed essential oil from the state of Rio de Janeiro showed α-humulene (α-caryophyllene) as the major compound.

There are few studies related to cytotoxic activity of essential oils from Salicaceae in tumor cell lines (Silva et al., 2008; Bou et al., 2013; Nikolic et al., 2014; Hayan et al., 2016). This data suggested that sesquiterpenes have cytotoxic activity, and indicated that further chemical and biological studies with other species from *Casearia* genus should be undertaken.

In recent years, the clonogenic assay is frequently used in cancer research laboratories to determine the survival and proliferation of tumor cells after experimental treatments (Hoffman, 1991; Tian et al., 2016). In these experiments, treatment with essential oil of *C. sylvestris* at 4.0 µg/ml reduced the colony-forming ability of A549 cells to 6% of the value obtained with the control. This effect could be due to interference of essential oil in the membrane integrity, DNA fragmentation or mitochondrial depolarization (Li et al., 2014; Ruan et al., 2015).

According to literature, the clonogenic assay is the method of choice to determine cell reproductive death after treatment with cytotoxic agents (Franken et al., 2006). The comet assay is widely used to evaluate the genotoxic potential in animal tissues, especially in studies on cancer therapy (Fairbairn et al., 1995; Dantas et al., 2002). According to other studies, the essential oils cause damages on tumor cell lines. For instance it has also been detected that the MCF-7 cell line is not able to repair the lesions on DNA, possibly due to the production of epoxides which induces DNA damage caused by essential oils, as well as by lipid peroxidation of essential oil rich in sesquiterpenes on V79 cells (Péres et al., 2009; Ortiz et al., 2016). There is evidence that DNA damages can be caused due to the epoxide formation by the induction of epoxide hydrolase 1 (EPHX1) responsible for the detoxification of exogenous chemicals (Ortiz et al., 2016).

In addition, this assay is extremely recommended when the toxicity of the agent tested is unknown, especially when there is no available data on cytotoxicity or genotoxicity (Tice et al., 2000). To the best of our knowledge, this is the first DNA damage study of the essential oil of *C. sylvestris* in a tumor cell line using two assays to detect potential harmful effects.

Conclusion

The essential oil from leaves of *C. sylvestris* exhibited potent DNA damage on A549 tumor cell line.

Conflict of interests

The authors have not declared any conflicts of interests.

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REFERENCES

Adams RP (2001). Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy. 3rd ed. Illinois: Allured Publishing. 456p.

Almeida MR, Darin JDC, Hernandes LC, Ramos MFS, Antunes LMG, Freitas O (2012). Genotoxicity assessment of Copaíba oil and its fractions in Swiss mice. Genet. Mol. Biol. 35(3):664-672.

Balbino EE, Dias MF (2010). Farmacovigilância: um passo em direção ao uso racional de plantas medicinais e fitoterápicos. Rev. Bras. Farmacogn. 20(6):992-1000.

Bou DD, Lago JH, Figueiredo CR, Matsuo AL, Guadagnin RC, Soares MG, Sartorelli P (2013). Chemical composition and cytotoxicity evaluation of essential oil from leaves of *Casearia sylvestris*, its main compound α-zingiberene and derivatives. Molecules 18(8):9477-9487.

Cestari MM, Lemos PMM, Ribeiro CAO, Costa JRMA, Pelletier E, Ferraro MVM, Mantovani MS, Fenocchio AS (2004). Genetic damage induced by trophic doses of lead in the neotropical fish hoplias malabaricus (Characiformes, Erythrinidae) as revealed by the comet assay and chromosomal aberrations. Genet. Mol. Biol. 27(2):270-274.

Dantas FJS, De Mattos JCP, Moraes MO, Viana ME, Lage CAS, Cabral-Neto JB, Leitão AC, Bernardo-Filho M, Bezerra RJAC, Carvalho JJ, Caldeira-de-Araujo A (2002). Genotoxic effects of stannous chloride ($SnCl_2$) in K562 cell line. Food. Chem. Toxicol. 40:1493-1498.

Esteves I, Souza IR, Rodrigues M, Cardoso LGV, Santos LS, Sertie JAA, Perazzo FF, Lima LM, Schneendorf JM, Bastos JK, Carvalho, JCT (2008). Gastric antiulcer and anti-inflamatory activities of the essentail oil from *Casearia sylvestris* Sw. J. Ethnopharmacol. 101:191-196.

Fairbairn DW, Olive PL, O'Neil KL (1995). The comet assay: a comprehensive review. Mutat. Res. 339:37-59.

Ferreira PMP, Bezerra DP, Silva JN, Costa MP, Ferreira JRO, Alencar NMN, Figueiredo IST, Cavalheiro AJ, Machado CML, Chammas R, Alves, APNN, Moraes MO, Pessoa C (2016). Preclinical anticancer effectiveness of a fraction from *Casearia sylvestris* and its component Casearin X: *in vivo* and *ex vivo* methods and miscroscopy examinations. J. Ethnopharmacol. 186:270-279.

Franken NAP, Rodermond HM, Stap J, Haveman J, Van Bree C (2006).

Clonogenic assay of cells *in vitro*. Nat. Protoc. 1:2315-2319.
Hayan G, Lijuan H, Shaoyu L, Chen Z, Ashrafi, MA (2016). Antimicrobial, antibiofilm and antitumor activities of essential oil of *Agastache rugosa* from Xinjiang, China. Saudi. J. Biol. Sci. 23(4):524-530.
Hoffman RM (1991). In vitro sensitivity assays in cancer: A review, analysis, and prognosis. J. Clin. Lab. Anal. 5:133-143.
Li WY, Ng YF, Zhang H, Guo ZD, Kwan YW, Leung GP, Lee SM, Yu PH, Chan SW (2014). Emodin elicits cytotoxicity in human lung adenocarcinoma A549 cells through inducing apoptosis. Inflammopharmacology. 22(2):127-134.
Maistro EL, Mota SF, Lima EB, Bernardes BM, Goulart FC (2010). Genotoxicity and mutagenicity of *Rosmarinus officinalis* (Labiatae) essential oil in mammalian cells *in vivo*. Genet. Mol. Res. 9(4):2113-2122.
Marquete R, Mansano VF (2013). A new species of *Casearia* (Salicaceae) from Brazil. J. Syst. Evol. 51:2013-2228.
Marquete R, Vaz ASF (2007). O gênero *Casearia* no Estado do Rio de Janeiro, Brasil. Rodriguésia 58:705-738.
Miyaji CK, Jordão BQ, Ribeiro LR, Eira AF, Cólus IMS (2004). Genotoxicity and antigenotoxicity assessment of shiitake (Lentinula edodes (Berkeley) Pegler) using the comet assay. Genet. Mol. Biol. 27(1):108-114.
Nikolic M, Glamoclija J, Ferreira ICFR, Calhelha RC, Fernandes A, Markovic T, Markovic D, Giweli A, Sokovic M (2014). Chemical composition, antimicrobial, antioxidant and antitumor activity of *Thymus serpyllum* L., *Thymus algeriensis* Boiss. and Reut. And *Thymus vulgaris* L. essential oils. Ind. Crops. Prod. 52:183-190.
Ortiz C, Morales L, Sastre M, Haskins WE, Matta J (2016). Cytotoxicity and genotoxicity assessment of Sandalwood essential oil in human breast cell lines MCF-7 and MCF-10A. J. Evid. Based. Complement. Altern. Med. 2016:13.
Pereira FG, Costa FB, Marquete R, May B, Falcão DQ, Mansur E, Moreira DL, Romanos MTV (2016). Anti-herpes activities of the pure and nanoemulsion of essential oil from leaves of *Casearia sylvestris* Sw. (SALICACEAE). Int. J. Green Herbal Chem. 5(2B):112-121.
Péres VF, Moura DJ, Sperotto ARM, Damasceno FC, Caramão EB, Zini CA, Saffi J (2009). Chemical composition and cytotoxic, mutagenic and egnotoxic activities of the essential oil from *Piper gaudichaudianum* Kunth leaves. Food Chem. Toxicol. 47(9):2389-2395.
Ruan Y, Hu K, Chen H (2015). Autophagy inhibition enhances isorhamnetin-induced mitochondria-dependent apoptosis in non-small cell lung cancer cells. Mol. Med. Rep. 12(4):5796-5806.
Silva SL, Chaar JS, Figueiredo PMS, Yano T (2008). Cytotoxic evaluation of essential oil from *Casearia sylvestris* Sw on human cancer cells and erythrocytes. Acta. Amaz. 38(1):107-112.
Sousa FG, Schneider FZ, Mendes CE, Moura NF, Denardin RBN, Matuo R, Mantovani MS (2007). Clastogenic and anticlastogenic effect of the essential oil from *Casearia sylvestris* Swartz. J. Essent. Oil. Res. 19:376-378.
Tian F, Han Y, Yan X, Zhong D, Yang G, Lei J, Li X, Wang X (2016). Upregulation of microrna-451 increase the sensitivity of A549 cells to radiotherapy through enhancement of apoptosis. Thorac. Cancer 7:226-231.
Tice RR, Agurell E, Anderson D, Burlison B, Hartmann A, Kobayashi H, Iyamae Y, Rojas E, Ryu JC, Sasaki YF (2000). Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ. Mol. Mutagen. New York. 35:206-221.
Tininis AG, Assonuma AA, Telascrea M, Perez CC, Silva MRSRM, Favoreto R, Cavalheiro AJ (2006). Composição e variabilidade química de óleo essencial de *Casearia sylvestris* Sw. Rev. Bras. Plantas Med. Botucatu. 8(4):132-136.
Tomazi LB, Aguiar P, Citadini-Zanette V, Rossato AE (2014). Estudo etnobotânico das árvores medicinais do Parque Ecológico Municipal José Milanese, Criciúma, Santa Catarina, Brasil. Rev. Bras. Plantas Med. 16(2):450-461.
Van Den Dool H, Kratz PD (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J. Chromatogr. A. A11:463-471.