

Cytotoxic evaluation of essential oil from *Casearia sylvestris* Sw on human cancer cells and erythrocytes

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ABSTRACT

Casearia sylvestris (Flacourtiaceae) is a plant popularly known as “guaçatonga” and it is used by indigenous population from South America (Brazil, Peru and Bolivia) in the treatment of several diseases, including cancer. Cytotoxic studies showed that it presents an interesting antitumoral potential due to the presence of casearins and the essential oil showed a high percentage of potent cytotoxic sesquiterpenes (β -caryophyllene and α -humulene). In this work, we verified that the essential oil of *C. sylvestris* presents a good selective cytotoxicity against HeLa, A-549 and HT-29 tumor cells (CD_{50} 63.3, 60.7 and 90.6 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively) when compared to non-tumoral cells Vero (CD_{50} 210.1 $\mu\text{g}\cdot\text{ml}^{-1}$) and mice macrophages (CD_{50} 234.0 $\mu\text{g}\cdot\text{ml}^{-1}$). The oil causes hemolysis in seven different kinds of erythrocytes, indicating that *C. sylvestris* must be used carefully. Besides, standard of β -caryophyllene and α -humulene were also tested and they showed similar cytotoxicity to the cytotoxicity presented in the oil, indicating that they might be responsible for the toxic effects that were observed in this study.

KEYWORDS: Cytotoxicity, *Casearia sylvestris*, Erythrocytes, Essential oil, MTT, Sesquiterpenes.

Avaliação citotóxica do óleo essencial de *Casearia sylvestris* Sw sobre células tumorais humanas e eritrócitos

RESUMO

A *Casearia sylvestris* (Flacourtiaceae) é uma planta popularmente conhecida como “guaçatonga” e é usada por povos indígenas da América do sul (Brasil, Peru e Bolívia) no tratamento de muitas doenças, incluindo câncer. Estudos citotóxicos mostraram que esta planta apresenta um possível e interessante potencial antitumoral devido à presença de moléculas chamadas casearinas. Além disso, a composição do óleo essencial mostrou uma alta concentração de sesquiterpenos de alto potencial citotóxico. Neste trabalho, nós verificamos que o óleo essencial da *C. sylvestris* apresentou uma boa citotoxicidade seletiva contra as linhagens de células tumorais HeLa, A-549 and HT-29 (CD_{50} 63,3, 60,7 e 90,6 $\mu\text{g}\cdot\text{ml}^{-1}$, respectivamente) quando comparada às células não-tumorais Vero (CD_{50} 210,1 $\mu\text{g}\cdot\text{ml}^{-1}$) e macrófagos de camundongos (CD_{50} 234,0 $\mu\text{g}\cdot\text{ml}^{-1}$). Além disso, o óleo causou hemólise em sete diferentes tipos de eritrócitos, indicando que a *C. sylvestris* precisa ser usada com cuidado. Também foram testados padrões de β -cariofileno e α -humuleno que mostraram citotoxicidade similar àquelas apresentadas pelo óleo, indicando que estes compostos podem ser os responsáveis pelos efeitos tóxicos que foram observados neste estudo.

KEYWORDS: Citotoxicidade, *Casearia sylvestris*, Eritrócitos, Óleo essencial, MTT, Sesquiterpenos.

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INTRODUCTION

Casearia sylvestris Sw (Flacourtiaceae) is a plant popularly known as “guaçatonga”, “chá-de-bugre” or “cafezinho-do-mato” and it is geographically distributed throughout Latin America (Lorenzi & Matos, 2002), also occurs in all extension of Brazilian territory (Felfili *et al.*, 2002; Barbosa, *et al.*, 2005; Hack, *et al.*, 2005). The Karajá Indians from Brazilian prepare a maceration of this plant bark to treat diarrhoea and the Shipibo-Conibo Indians of Peru use a decoction of the bark for diarrhea, chest colds and flu. Other Indian tribes in Brazil smash the roots or seeds of guaçatonga to treat wounds and topical leprosy (Chiappeta, 1983; Taylor, 2002). The indigenous populations throughout the Amazon rainforest have used guaçatonga for a long time as a snakebite medicine (Ruppelt, 1991; Alves, 2000; Mors *et al.*, 2000).

The literature have some studies that corroborate some medicinal properties of *C. sylvestris* as being anti-inflammatory (Raslan *et al.*, 2002), antiulcerous (Basile *et al.*, 1990; Esteves *et al.*, 2005), anticancer (Morita *et al.*, 1991; Mans *et al.*, 2000) and antimicrobial (Da Silva, 2006). Nowadays, *C. sylvestris* is being used as an active principle in creams against herpes and it is also being commercialized in plants combinations used for make tea and infusions with phytotherapeutic purposes (Alves, 2000; Beutler *et al.*, 2000; Menezes *et al.*, 2004).

Mosaddik *et al.* (2004) showed the cytotoxic activity of *C. Costulata*, *C. Grewiifolia*, *C. Grayi* and *C. Multinervosa* on P388 lymphoblast cells of mouse. Orberlies *et al.* (2002) showed that alcohol extracts of *C. sylvestris* are cytotoxic against macrophages J774, however Maistro *et al.* (2004) showed that the alcohol extracts of *C. sylvestris* were not genotoxic against HTC (hepatoma tissue cells) and VT79 (Chinese hamster lung) cells.

Esteves *et al.* (2005) have determined recently the composition of the essential oil obtained from *C. sylvestris* leaves and verified that the major component is the bicyclogermacrene (40.9%), a component with low antitumoral potential. Nevertheless, a high percentage of this essential oil (around 17.5%) is constituted by two sesquiterpens, which cytotoxic activity is very potent and well characterized: β -caryophyllene (13.8%) and α -humulene (3.7%) (Duh *et al.*, 1999; Tatman *et al.*, 2002; Sibanda *et al.*, 2004; Sylvestre *et al.*, 2005; Sylvestre *et al.*, 2006; Hou *et al.*, 2006; Xiao *et al.*, 2006).

This work describes the evaluation of the *C. sylvestris* essential oil cytotoxicity against tumor cells (HeLa, A-549 and HT-29 cell lines). In addition, to contribute to the safe usage of this plant as a phytopharmaco, the *C. sylvestris* leaves essential oil was tested against non tumoral cells (Vero cell, mice macrophages and seven different types of erythrocytes). Since the essential oil of this plant is rich in β -caryophyllene and α -humulene (cytotoxic sesquiterpens against tumoral

cells), the results obtained with *C. sylvestris* leaves essential oil were compared to the standards of these compounds that were also experimentally evaluated.

MATERIAL AND METHODS

COLLECTION OF PLANT MATERIAL

The fresh leaves of *C. sylvestris* were collected in Campinas city, S.P., Brazil, in 2005 March. The specimen was identified by Prof. A.M. Schimidel and a voucher was deposited under the number UEC 118743 in the herbarium of Unicamp Biological Institute.

EXTRACTION OF THE ESSENTIAL OIL

The essential oil was obtained from 750g of leaves that were subdued to hydrodistillation for 4 hours in a modified Clevenger device resulting in a yellow essential oil (yield of 0.98%). The oil was dried with anhydrous sodium sulphate and stored at -20°C until use (Da Silva *et al.*, 2006; Da Silva *et al.*, 2007).

OIL STANDARDIZATION BY GC-MS

In order to characterize and standardize the essential oil, it was analyzed through gas chromatography (CG) with a Hewlett-Packard (HP) instrument model 5890 equipped with a flame ionization detector (FID) and a SE-54 glass column (25 m \times 0.25 mm i.d., film thickness 0.25 μ m). The carrier gas was helium at 1.0 ml.min⁻¹. The oven temperature was programmed from 50 to 250 °C at 4 °C/min rate. Injector and interface temperatures were 220 and 250 °C. Gas chromatography-mass spectrometry (GC-MS) analyses were performed by using a Hewlett-Packard model 5880 (quadruple analyzer), operated in the electron impact mode (70 eV), coupled to a Hewlett-Packard mass spectrometer model 5897A. High purity helium was used as carrier gas at a linear velocity of 1 ml.min⁻¹. The temperature conditions were the same used at CG analysis. The retention indexes (RI) were calculated through the Kovats index. In this determination was utilized a mixture of alkane standards C₈-C₁₆ added to the essential oils solution, and injected in the gas chromatograph in the same conditions of the chromatographic analysis. The identifications were carried out by comparison with MS literature data and by comparison of their RI values with those of pure standards and confirmed with the aid of RI from published sources whenever necessary (Davies, 1990; Massada, 1995; Adams, 1995).

SESQUITERPENES STANDARDS TESTED

The standards of β -caryophyllene and α -humulene used in the comparison of the cytotoxic activity of the *C. sylvestris* leaves essential oil were supplied by Sigma (Co St. Louis, MO).

CELL CULTURE

HeLa cell lines (human cervical carcinoma), A-549 (human lung carcinoma), HT-29 (human colon adenocarcinoma) and Vero (monkey kidney) were obtained from the American Type Culture Collection (ATCC). The cells grew in RPMI 1640 supplemented with 10% fetal calf serum 1% (w/v) glutamine, 100 U.ml⁻¹ penicillin, 100 µg.ml⁻¹ streptomycin and 5 µg.ml⁻¹ amphotericin B. The cells were cultured in a humidified atmosphere at 37°C in 5% CO₂ (Da Silva *et al.*, 2007). The macrophages were isolated from mice and kept according to the methodology described by Tseng *et al.* (2006).

IN VITRO ASSAY CYTOTOXIC ACTIVITY

For the *in vitro* cytotoxicity assay, the cells were washed with phosphate-buffered saline (PBS) free of magnesium and calcium. After PBS decantation, the cells were detached by addition of 0.025% trypsin-EDTA and PBS to a final volume of 50 ml and centrifuged. The pellet was suspended in 10 ml of a medium to obtain a single cell suspension. The density of viable cells was determined by Trypan blue exclusion in a hemocytometer and the preparation was diluted within a medium to yield previously determined optimal plating densities for cells.

Before the assay, 5 x 10⁴ cells / well were seeded on 96-well plates and the suspension was incubated 24 hours at 37°C to cell attachment. After 24 hours, the cells were treated with the essential oil and sesquiterpenes. The oil was dissolved in ethanol and a serial of doubling essential oil dilution was added to five replicate wells, over the range of 600 - 0.6 µg.ml⁻¹ against all cell lines and macrophages. Sesquiterpenes were also dissolved in ethanol and tested to five replicate wells, but over the range of 200 - 0.2 µg.ml⁻¹. The final concentration of ethanol in the culture medium was kept at 0.5% (v/v) to avoid solvent toxicity. The activities of the essential oil and sesquiterpenes were considered according to the survival of 50% or less cells after an exposure time of 72 hours. The cell culture used as control received only 0.5% ethanol at final concentration.

The cytotoxicity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After an exposure time of 72 hours, the medium was removed and then, MTT assays were performed using the cell titer kit (Promega Corp., USA). On a 96-well plate, 20 mL of MTT (5 mg.ml⁻¹) in PBS was incubated with cells for 2 hours at 37°C. After this period, the medium containing MTT was removed and 100 ml of acidified isopropanol (0.04 mol.l⁻¹ HCl) was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, model 3550, USA). The cell viability was expressed with respect to the absorbance of the control wells, which were considered

as 100% of absorbance. The cytotoxicity is expressed as Cytotoxic Dose, the concentration of the substance inhibiting cell growth by 50% (CD₅₀). Three replicate plates were used to determine the cytotoxicity of each sample (Stavri *et al.*, 2005; Hou *et al.*, 2006; Xiao *et al.*, 2006; Da Silva *et al.*, 2007).

HEMOLYTIC ACTIVITY

The hemolytic activity was assayed by incubating the essential oil or sesquiterpenes with of 2% erythrocytes (sheep, bovine, horse, guinea pig, rabbit, chicken, human) washed three times with PBS (phosphate buffer saline), pH 7.2 for 1 hour at 37°C in round-bottom 96-well plates. The oil was dissolved in DMSO (dimethyl sulfoxide) and a serial of double essential oil dilution was added to five replicate wells, over the range of 600 - 0.6 µg.ml⁻¹ against all erythrocytes. Sesquiterpenes were also dissolved in ethanol and tested to five replicate wells, but over the range of 400 - 0.2 µg.ml⁻¹. The control wells received only 0.2% DMSO and its final concentration in all assays were kept constant to avoid solvent toxicity (Bhakdi *et al.*, 1986). The hemolytic activity was expressed as the Maximum oil Concentration (or sesquiterpenes) that does not cause Hemolysis (MCnH). Three replicate plates were used to determine the hemolytic activity of each sample.

STATISTICAL ANALYSIS

Data are reported as the mean ± S.D. for at least three replicates. Statistical analysis were performed using the Student t-test, with significance level set at P < 0.05.

RESULTS

The Table 1 shows the standardization of essential oil through GC-MS. The major compounds observed in the essential oil chemical composition used in this work were: bicyclogermacrene (43.6%), β-caryophyllene (18.1%), α-humulene (4.7%), spathulenol (15.9%), germacrene B (5.2%), α-humulene (4.7%), α-pinene (4.0%), germacrene D (3.9%), globulol (3.0%), α-muurolol (2.7%). The oil used on this study presents a high percentage of two sesquiterpenes with high cytotoxic activity: β-caryophyllene (18.1%) and α-humulene (4.7%) (Duh *et al.*, 1999; Tatman *et al.*, 2002; Silvestre *et al.*, 2006; Hou *et al.* 2006; Xiao *et al.*, 2006).

The results of cytotoxic and hemolytic activities in the *in vitro* assay were summarized in Tables 2 and 3. From Table 2, it is possible to verify that the oil extracted from *C. sylvestris* leaves and the sesquiterpenes tested presented some degree of toxic activity against all the tested cells. It was also observed that the tumor cells A-549, HeLa and HT-29 were much more sensitive to the essential oil of guaçatonga (CD₅₀ 63.3, 60.7 and 90.6 µg.ml⁻¹, respectively) when compared to the non tumoral Vero cells (CD₅₀ 310.1 µg.ml⁻¹) and

Table 1 - Chemical composition of essential oil from *C. sylvestris* used in this work.

Compounds	RI ^a	%
α -pinene	938	4.0
Linalool	1111	0.7
Pulegone	1235	0.5
α -elemene	1343	1.1
α -copaene	1380	0.8
β -bourbonene	1388	0.5
β -elemene	1390	1.6
β -caryophyllene	1426	18.1
χ -elemene	1427	0.4
β -gurjunene	1430	0.4
α -humulene	1456	4.7
muurolene	1471	0.5
germacrene D	1482	3.9
bicyclogermacrene	1498	43.6
germacrene B	1501	5.2
calamenene	1521	2.3
δ -cadinene	1524	0.8
spathulenol	1574	15.9
Globulol	1582	3.0
α -muurolol	1644	2.7

^a Retention indices on SE-54 column.

mice macrophages (CD₅₀ 334.0 $\mu\text{g}\cdot\text{m}^{-1}$). The same pattern of results was observed in the tests using the compounds β -caryophyllene and α -humulene, it means, the cells A-549, HeLa and HT-29 are more sensitive to these sesquiterpens than the Vero cells and mice macrophages.

The hemolytic activity expressed in MCnH (Maximal Concentration not causing Hemolysis - $\mu\text{g}\cdot\text{ml}^{-1}$) is shown in

Table 3. In all cases, erythrocytes from the different species have showed various degrees of hemolysis in the presence of both essential oil extracted from *C. sylvestris* leaves and the tested sesquiterpens. The human and bovine erythrocytes were the most sensitive to the presence of the essential oil (MCnH 156.2 and 158.3 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively), while the chicken erythrocytes were the less sensitive ones (MCnH 583.7 $\mu\text{g}\cdot\text{ml}^{-1}$). In relation to the sesquiterpens, the human, bovine and sheep erythrocytes were more sensitive (see Table 2).

CONCLUSIONS

The differential cytotoxicity of *C. sylvestris* against tumoral cells A-549, HeLa and HT-29 shows that the use of the plant against different types of cancers might present positive results, since the leaves essential oil presented low cytotoxicity against non tumoral cells (Vero cells and mice macrophages). Moreover, the tests realized using two sesquiterpens found in guaçatonga (β -caryophyllene and α -humulene) presented similar results to the ones verified for the plant essential oil, indicating that they might be responsible for the cytotoxic action verified. In fact, the sum up of β -caryophyllene and α -humulene fractions of *C. sylvestris* represents a high percentage of the essential oil total composition used in this study (around 22.8%), and it was possible to suppose that the cytotoxic effects against tumoral cells (A-549, HeLa and HT-29) observed in the action of *C. sylvestris* essential oil can be related to the presence of both substances on this plant's leaves. These sesquiterpens have already been described as cytotoxic components of other plant oils (Sibanda *et al.*, 2004; Sylvestre *et al.*, 2005; Hou *et al.*, 2006; Sylvestre *et al.*, 2006).

Table 2 – Cytotoxic activity of essential oil from leaves of *C. sylvestris* Sw, β -caryophyllene and α -humulene in mammalians cells lines (CD₅₀ - $\mu\text{g}\cdot\text{ml}^{-1}$)^a.

	Vero	Macrophages	A-549	HeLa	HT-29
Volatile oil	310.1 \pm 1.7	334.0 \pm 2.1	63.3 \pm 1.1	60.7 \pm 6.2	90.6 \pm 8.4
β -caryophyllene	88.3 \pm 4.1	107.3 \pm 9.1	29.3 \pm 1.4	21.8 \pm 0.9	40.9 \pm 0.7
α -humulene	129.9 \pm 0.3	159.0 \pm 3.2	18.9 \pm 0.4	35.2 \pm 1.3	24.8 \pm 1.4

^aAverage of three independent determinations; five replicates; values are mean \pm S.D.

Table 3 – Hemolytic activity of volatile oil from leaves of *C. sylvestris* Sw, β -caryophyllene and α -humulene in mammalians erythrocytes (MCnH $\mu\text{g}\cdot\text{ml}^{-1}$)^a.

	Human	Bovine	Sheep	Horse	Guinea pig	Rabbit	Chicken
volatile oil	156.2 \pm 0.5	158.3 \pm 0.7	297.3 \pm 1.2	288.1 \pm 1.3	356.2 \pm 0.4	350.1 \pm 0.3	583.7 \pm 3.2
β -caryophyllene	96.3 \pm 1.7	108.9 \pm 2.3	107.6 \pm 1.7	243.4 \pm 2.0	328.3 \pm 0.9	275.7 \pm 2.7	396.6 \pm 0.6
α -humulene	98.9 \pm 0.9	99.7 \pm 0.6	121.0 \pm 1.4	275.4 \pm 3.7	285.5 \pm 2.2	338.3 \pm 1.5	> 400

^aAverage of three independent determinations; five replicates; values are mean \pm S.D.

These results are important for the characterization of the cytotoxicity action of *C. sylvestris*, since most of the investigations related to the cytotoxic activity of guaiatonga are mainly associated to diterpens (casearins) isolated from alcoholic extracts of the plant (Morita *et al.*, 1991; Carvalho *et al.*, 1998; Beutler *et al.*, 2000; Orberlies *et al.*, 2002; Maistro *et al.*, 2004; Mosaddick *et al.*, 2004). The selective action of β -caryophyllene and α -humulene in the *C. sylvestris* essential oil against tumor cells associated to casearins isolated from alcoholic extracts might help in the explanation of the anticancer activity popularly associated to the plant.

However, in opposition to the studies of Basile *et al.*, (1990) which verified that the *C. sylvestris* leaves alcoholic extracts caused low acute toxicity, we concluded that the use of solutions containing high content of essential oil from leaves of guaiatonga must be carried out carefully. In fact, from Table 3, it was possible to verify that all the tested erythrocytes were, in some degree, sensitive to the essential oil of *C. sylvestris*. The human and bovine erythrocytes were the most sensitive to the essential oil, indicating that, for the therapeutic use of the plant, it is necessary to observe the quantity of oil that reaches the bloodstream. Since the erythrocytes have presented the same sensitivity to both β -caryophyllene and α -humulene, we can attribute to the sesquiterpens, at least in part, the toxic effects observed.

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