

Artigo

Cytotoxicity Assessment of *Siparuna cymosa* Essential Oil in the Presence of Myeloid Leukemia Cells

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Avaliação Citotóxica do Óleo Essencial de *Siparuna cymosa* Frente a Células de Leucemia Mielóide

Resumo: Nesse trabalho é apresentado a caracterização química do óleo essencial de *Siparuna cymosa* (Siparunaceae), espécie endêmica da Mata Atlântica, bem como sua avaliação citotóxica frente a linhagem de células tumorais. O óleo foi extraído das folhas secas por hidrodestilação, caracterizado (CG-EM) e submetido a teste de citotóxica através do método colorimétrico usando brometo de 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-tetrazólio (MTT) em linhagens de LMA, Leucemia Mielóide Aguda, (THP-1) e LMC, Leucemia Mielóide Crônica (K562). O teor médio de óleo foi de 2,11%, tendo como composto majoritário α -bisabolol (68,9%) e, em quantidade significativas, *p*-cimen-9-ol (7,9%) e espatulenol (3,7%). Os valores de IC_{50} foram de $25,44 \pm 1,55 \mu\text{g mL}^{-1}$ (LMC) e $30,88 \pm 2,45 \mu\text{g mL}^{-1}$ (LMA). O óleo essencial apresenta moderada ação citotóxica frente as células leucêmicas, destacando-se a LMA que obteve maior índice de seletividade


Keywords: Siparunaceae; análise CG-EM; bisabolol; THP-1; K562.

Abstract

This work presents the chemical characterization of the essential oil obtained from the *Siparuna cymosa* (Siparunaceae), an endemic species of the Atlantic Rainforest, as well as its cytotoxicity assessment in the presence of tumor cells. The oil was extracted from dry leaves by hydrodistillation and characterized (GC-FID, GC-MS); and its cytotoxicity was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric method in AML, Acute Myeloid Leukemia (THP-1) and CML, Chronic Myeloid Leukemia (K652) cell lines. The average oil content was 2.11% with the major compound being α -bisabolol (68.9%) and, in significant amounts, *p*-cymen-9-ol (7.9%) and spathulenol (3.7%). The IC_{50} obtained were the $25.44 \pm 1.55 \mu\text{g mL}^{-1}$ (CML), and $30.88 \pm 2.45 \mu\text{g mL}^{-1}$ (AML). The essential oil exhibits moderate cytotoxic action in the presence of leukemia cells, especially AML, for which the selectivity index was higher.

Palavras-chave: Siparunaceae; GC-MS analysis; bisabolol; THP-1; K562.

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Cytotoxicity Assessment of *Siparuna cymosa* Essential Oil in the Presence of Myeloid Leukemia Cells

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1. Introduction

Leukemias are malignant neoplasms in young hematopoietic cells that result in the unregulated proliferation of mutated cells that exhibit changes in differentiation and apoptosis mechanisms. Leukemias are grouped based on the affected cell line, and may be lymphoid or myeloid. They may also be classified as acute or chronic, in accordance with their clinical manifestation.¹

Chronic myeloid leukemia (CML) is a myeloproliferative disease of the bone marrow, a type of cancer, resulting from the reciprocal translocation between the long arms of chromosomes 9 and 22, which give rise to the Philadelphia chromosome (Ph).¹ Acute myeloid leukemia (AML) is characterized by the clonal expansion of immature cells, resulting in the abnormal build-up of these cells in the bone marrow, leading to a reduction in other cells' production, such as red blood cells, leukocytes and platelets. The build-up of these immature cells,

in most cases, occurs in the blood and may spread to other body parts, such as lymph nodes, spleen, liver, testicles, and central nervous system.² The onset of resistance to chemotherapy represents a great obstacle to improving the response to treatment and patient survival.

The discovery of some isolated antitumor agents in plant species, such as vincristine, vinblastine, or taxol is a motivation for researching new plant substances with cytotoxic action for tumor cells. Essential oils may exhibit cytotoxic action and some examples can be found from the species of *Casearia sylvestris*,³ *Philippine calamansi*,⁴ *Potentilla descolorir*,⁵ *Chenopodium ambrosioides*,⁶ and *Pistacia khinjuk*.⁷

The different plant species studied for medicinal purposes include the *Siparuna* genus. In 2003, despite this genus being recognized by many taxonomists as belonging to the Monimiaceae family, it was segregated to the Siparunaceae family by the APG II, Angiosperm Phylogeny Group. The *Siparuna* genus, native of South America, consists of approximately 65 species.⁸

Ethnopharmacological studies reveal that some species have been used popularly, being the most frequently studied one the *S. guianensis* species. This species' leaves are used to treat stomach disorders, headaches and rheumatism, chills, fevers, blood pressure, rheumatic diseases, and cramps.⁸ Its extract is used as an insecticide.⁸ It is also used as an anxiolytic drug by South American indigenous peoples and by riparian populations.⁹ Other studies report the use of *S. apiosyce* in treating dyspepsia, flatulence, coughs, bronchitis, and laryngitis, skin diseases, fevers, flus, headaches, rheumatism,¹⁰ and snakebites.¹¹ *S. thecaphora* is used to treat colds and headaches.¹² *S. pauciflora* leaves are also used by indigenous peoples to treat fevers.¹³

Fonseca *et al.* (2008) reported on the antibacterial activity of the *S. sessiliflora* leaves' alkaloid fraction in the presence of *Bacillus subtilis*. *S. arianae* leaves' raw extract exhibited antibacterial activity in the presence of *Mycobacterium malmoense*.¹⁴

Siparuna species' essential oils are not frequently studied. To contribute to the search for substances with cytotoxic action in the presence of AML (THP-1) and CML (K562), this article shows the extraction and characterization of the *S. cymosa*, as well as its cytotoxicity assessment in the presence of these cells. *S. cymosa* is an endemic species from southern Bahia, Brazil, an Atlantic

rainforest region. Our research group was the first to study this species

2. Methodology

2.1. Plant material

S. cymosa Tolm. species leaves were collected in May 2017, in the city of UNA (GPS 15° 12'34.2"S and 39° 03'36.4"W), Bahia, Brazil. The taxonomical identification of the species was done by botanist Luiz Alberto Mattos and the specimen voucher was deposited in the Herbarium of the State University of Santa Cruz, registered under number 16332. The leaves were dried in a forced-ventilation greenhouse at 40°C for 2 days.

2.2. Essential oil extraction and chemical analysis

The essential oil was extracted from 30 g of dry leaves that were cured in 1.5 liters of distilled water, using a Clevenger apparatus, for 90 minutes. The essential oil was separated from the hydrolate using the extraction with dichloromethane, subsequently dried with anhydrous sodium sulfate, and concentrated. The content was expressed in percentages, based on the ratio between the obtained oil mass and the used dry plant material (w/w), in triplicates. The oil was stored at -10°C.

The quantitative analysis of volatile oil was obtained by means of gas chromatography with flame ionization detector (GC-FID) using the gas chromatography system (Varian, Chrompack, CP-3800), equipped with fused silica capillary column VF5-ms (30m X 0.25 mm) with 5% phenyl and 95% dimethylpolysiloxane stationary phase (0.25µm film thickness), using 5.0 helium as the carrier gas, with a flow at 1.2 ml min⁻¹ (12 psi). Injector and detector temperatures were 250 and 280°C, respectively. A 1.0µl solution in 10% chloroform was injected, in split mode (1:10). The column temperature started at 60°C, with an 8°C per minute increase until it reached the temperature of 260°C, at which it was maintained for 5 minutes. The analysis time was 30 minutes. Component quantification present in the oil was obtained by electronic integration of the peaks detected in the FID by normalization, in triplicates.

Qualitative analysis of the essential oil was done by GC-MS, using the Varian (Chromopack Saturn 2000/2000) chromatography system equipped with the same capillary column and under the same conditions adopted for the GC-FID analysis. Helium 6.0 was used as the carrier gas, with a flow at 1.0 ml min^{-1} (10 psi). An ion trap analyzer was used, with transferline, manifold, and trap temperatures at 280°C , 50°C , and 150°C , respectively. The mass range analyzed was from 60 to 450 dalton. To identify the substances, present in the essential oil, the fragmentation patterns observed in the mass spectra were compared to the present study results in a NIST 11.0 database, and retention rates obtained with the injection of a $\text{C}_8\text{-C}_{26}$ (Sigma-Aldrich-USA) hydrocarbon mix, under the same analytical conditions, were compared to data from the literature.¹⁵

2.3. Artemia salina lethality assay

Artemia salina nauplius larvae were placed in a saline solution, under artificial lighting for 48 h, for eclosion. After this time, 10 microcrustaceans were counted and placed in transparent containers with a 5 mL resulting solution consisting of sea water, 1 mL Tween 80 at 5%, and five different concentrations of essential oil (1000 - 500 - 300 - 100 - 50 $\mu\text{g mL}^{-1}$), each one formed from 5 replicates. Sea water and a 5% Tween 80 solution were used as control in triplicates. A new count was performed 24 hours after the microcrustaceans were added in the present of light in order to determine the number of live and dead microcrustaceans at each concentration level. The interpolation method, in which the percentage of live and dead microcrustaceans is correlated, based on concentration, was used as the evaluation parameter to determine LC_{50} .

2.4. Cytotoxicity assay

The cytotoxic activity assessment used the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide colorimetric assay (MTT, Sigma-Aldrich) with an Acute Myeloid Leukemia cell line (ATCC TIB-202, THP-1) and a Chronic Myeloid Leukemia cell line (ATCC CRL-3344, K562). The cells were placed on 96-well plates (2×10^5 cells per well) and incubated for 24 hours at 37°C in a humid atmosphere, with 5% CO_2 . After 24 h,

the wells were washed with culture medium (RPMI-1640 + heat-inactivated fetal bovine serum at 20% + 2 m mol L^{-1} L-glutamine) and incubated with samples at 0.10 to $100 \mu\text{g mL}^{-1}$ concentrations. After a 48-hour incubation period, the plates were treated with MTT (5 mg mL^{-1}). Colorimetric measurements were done at 550 nm, using the Spectramax M5e microplate reader. The cytarabine and imatinin mesylate were used as controls for THP-1 and K562, respectively. All experiments were performed in triplicates. The results were expressed by the IC_{50} values (drug concentration that reduced the cell viability at 50%). The IC_{50} were calculated using the software OriginPro 8.0.

The selectivity index (SI) corresponds to the essential oil IC_{50} value in the presence of normal cell lines divided by the essential oil IC_{50} value in the presence of neoplastic cell lines – $\text{SI} = \text{normal cell } \text{IC}_{50} / \text{neoplastic cell } \text{IC}_{50}$.¹⁶

3. Results and Discussions

3.1. Essential oil

The average essential oil content was $2.11 \pm 0.09\%$; therefore, if compared to the oil contents for other *Siparuna* species, the *S. cymosa* exhibits high oil content. The *S. guianensis* exhibited essential oil content of 0.13 – 0.24%, for leaves, and of 0.04 – 0.09%, for branches.¹⁷ *S. thecaphora* dry leaves exhibited an oil content of 0.12%,¹⁸ while the *S. eggersii* fresh leaves exhibited a content of 0.19%.¹⁹

The essential oil chromatography analysis allowed to identify 20 components, representing 97.0% of total volatiles that could be divided into monoterpenes (10.7%), phenylpropanoids (1.3%), sesquiterpenes (3.7%), and oxygenated sesquiterpenes (81.3%), Figure 1 and Table 1. The α -bisabolol (68.3%) is the primary major component in the oil; while p-cymen-9-ol (7.9%) and spathulenol (3.7%) have also been identified in significant amounts.

The α -bisabolol has a 222 g mol^{-1} molecular mass, with base peak at m/z 109 g mol^{-1} . This compound was also identified in the *S. guianensis* essential oil.²⁰ The compound spathulenol has a molecular mass of 220 g mol^{-1} with a base peak at m/z 119 g mol^{-1} . Its presence was also reported in essential oils extracted from *S. thecaphora*

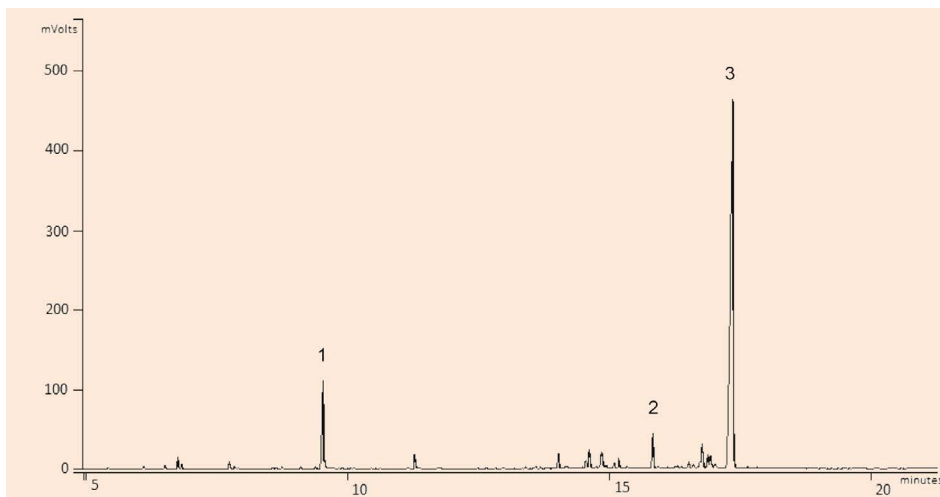


Figure 1. Chromatogram (GC-FID) of the essential oil extracted from *Siparuna cymosa* leaves, highlighting the major substances: 1: *p*-Cymen-9-ol; 2: Spathulenol; 3: α -Bisabolol

Table 1. Chemical composition of the *Siparuna cymosa* essential oil

Substances	RI (Exp)	RI (Lit)	Relative area (%)
3- δ -Carene	1010	1011	0.5 \pm 0.0
<i>o</i> -Cymene	1027	1027	1.1 \pm 0.2
<i>p</i> -Cymene	1031	1026	0.6 \pm 0.1
<i>p</i> -Menta-2,4 (8)-diene	1085	1086	0.6 \pm 0.1
<i>p</i> -Cymen-9-ol	1195	1206	7.9 \pm 0.6
Safrole	1292	1285	1.3 \pm 0.2
<i>epi</i> -Cubenol	1496	1493	0.8 \pm 0.1
β -Bisabolene	1503	1509	1.7 \pm 0.1
Cubebol	1517	1514	2.0 \pm 0.2
δ -Cadinene	1522	1524	0.6 \pm 0.0
γ -(E)-Bisabolene	1536	1531	0.5 \pm 0.0
Germacrene-B	1542	1556	0.9 \pm 0.1
Spathulenol	1581	1576	3.7 \pm 0.3
1- <i>epi</i> -Cubenol	1630	1628	0.7 \pm 0.0
Muurolo-4,10(14)-dien-1-- β -ol	1636	1631	0.6 \pm 0.0
Cubenol	1645	1642	0.8 \pm 0.0
α -Cadinol	1650	1653	2.3 \pm 0.0
α -Bisabolol oxide-B	1657	1655	1.4 \pm 0.0
β -Bisabolol	1670	1671	0.7 \pm 0.0
α -Bisabolol*	1699	1686	68.3 \pm 1.8
Monoterpenes		10.7	
Phenylpropanoids		1.3	
Sesquiterpenes		3.7	
Oxygenated sesquiterpenes		81.3	
Total identified (%)		97.0	

RI (Exp): Retention Index calculated based on the pattern injections C₈ – C₂₆; RI (Lit.) Literature Retention Index;¹⁵ Relative percentage based on the normalization of the chromatographic peaks; n = 3; *confirmed with authentic standard

leaves,¹² *S. eggerssi* leaves,¹⁹ and *S. guianensis* leaves,²¹ as well as from the *S. thecaphora* fruit.¹² The compound *p*-cymen-9-ol has a molecular mass of 150 g mol⁻¹ with a base peak at *m/z* 134.9 g mol⁻¹. This is the first time this compound has been reported in essential oils from species of this genus.

3.2. Artemia salina lethality assay

The essential oil exhibited LC₅₀ of 29.00 µg mL⁻¹, which is considered to be lethal in the presence of *A. salina*.²² Studies have shown the correlation between the lethality test in the presence of *A. salina* and the *in vitro* growth inhibition of tumor cells; recognizing this experiment, with simple and easy execution, as a preliminary evaluation form for the cytotoxicity assessment of substances in the presence of antitumor cells.²²

3.3. Cytotoxicity assay

Figure 2 shows the results of cytotoxicity assays for MTT tests in THP-1 and K562 cell lines. The control substances (chemotherapeutics) used in this study were cytarabine (THP-1) and imatinib mesylate (K562). The IC₅₀ values obtained for the essential oil in K562 (30.88 ± 2.45 µg mL⁻¹) and THP-1 (25.44 ± 1.55 µg mL⁻¹) were very similar and lower than 30.0 µg mL⁻¹, indicating cytotoxic activities.^{23,24}

The essential oil exhibited a PBMC (Peripheral Blood Mononuclear Cells) cytotoxic value of 80.20 ± 4.11 µg mL⁻¹, which is not considered

cytotoxic in the presence of these normal cells. The PBMC values for cytarabine and imatinib mesylate were of 58.70 ± 3.76 and 69.63 ± 3.13 µg mL⁻¹, respectively. The SI on the THP-1 cell line was 3.15 for the essential oil while the cytarabine index was 4.62. On K562 cells, the SI was 2.60 for the essential oil, whereas the imatinib index was 6.63. Based on these results, despite chemotherapeutics shows a better index than the essential oil analyzed here, the essential oil exhibits actions on both types of tumor cells.

The α-bisabolol, primary component of the essential oil exhibits such activities as antimutagenic, anti-inflammatory, antifungal, antibacterial, and gastroprotective action.^{25,26}

This compound also exhibited cytotoxic activity, in glioma tumor cell lines in mice, and it was quite selective.²⁷ In addition, it has cytotoxic activity in the presence of other tumor cell lines, such as melanoma, breast adenocarcinoma, liver carcinoma, lung carcinoma, pancreatic cancer, acute leukemia, and acute myeloid leukemias, resulting, in all of these cases, in death by apoptosis.²⁸

Studies conducted by Alcântara *et al* (2010) show spathulenol action, also present in the essential oil, as a bactericide, in addition to exhibiting moderate cytotoxic activity in the presence of KB type cells (oral carcinoma).²⁹

In addition to providing great yields, the *S. cymosa* oil, based on our biological assessment studies, may exhibit other activities, leading us to proceed with studies about the species.

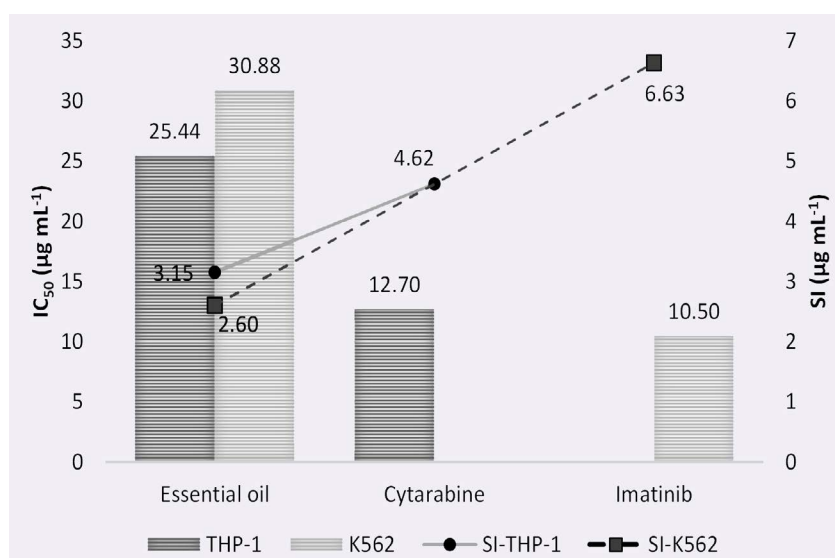


Figure 2. Cytotoxicity assay data for the species *Siparuna cymosa* essential oil. THP-1 and K562: Cell lines; SI: selectivity Index., Cytarabine and Imatinib mesylate: control substance for THP-1 and K562, respectively

4. Conclusion

The species studied herein is promising as a source of essential oil rich in α -bisabolol, being of great interest in the pharmaceutical and cosmetic areas. The essential oil presented high lethality against *A. salina*. In addition, the results indicated cytotoxic activity against the AML and CML tumour cell lines, with selectivity index higher for AML cells. Thus, the study of the *S. cymosa* species of the south region of Bahia becomes promising, since this work presented interesting results, and not yet recorded in the literature.

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