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Artigo

The Cytotoxic Effect of Extracts Obtained from *Cecropia catharinensis* Cuatrec (Urticaceae)

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Efeito Citotóxico de Extratos Obtidos de *Cecropia catharinensis* Cuatrec (Urticaceae)

Resumo: Os produtos naturais derivados de plantas continuam a ser uma importante fonte de substâncias biologicamente ativas para o tratamento do câncer. A ação citotóxica do gênero *Cecropia* ainda é pouco investigada, e a espécie *C. catharinensis* é aqui avaliada pela primeira vez. Este estudo relata a composição fitoquímica das folhas de *C. catarinensis,* além de sua atividade inibitória, *in vitro,* no crescimento celular de três linhagens de células tumorais humanas: adenocarcinoma de mama (MCF-7), células não pequenas de cancro de pulmão (NCI-H460) e melanoma (A375-C5), pelo extrato bruto (CME) e duas frações semi-purificadas (CME1 and CME2). O extrato bruto CME foi obtido por extração exaustiva das folhas secas de *C. catarinensis*. A fração de baixa polaridade (CME1) foi obtida a partir de CME pelo tratamento com diclorometano e acetato de etila, e a fração polar (CME2) corresponde ao resíduo insolúvel obtido no referido tratamento. Os efeitos do extrato bruto e das frações semi-purificadas foram avaliados sobre o crescimento celular de linhagens de células tumorais de acordo com os procedimentos adotados pelo Instituto Nacional do Câncer (NCI, USA). Os resultados foram expressos como valores médios de IC_{50} ($\mu g.mL^{-1}$) ± SD dos três experimentos independentes realizados em duplicata. CME inibiu o crescimento celular das três linhagens utilizadas nos ensaios biológicos. A fração menos polar CME1 apresentou a maior atividade inibitória entre as três amostras testadas. A fração polar CME2 mostrou ser moderadamente ativa contra as linhagens MCF-7 e NCI-H460 e foi inativa sobre as células A375-C5. Os resultados indicaram que a espécie *C. catarinenses* é uma potencial fonte de substâncias com ação citotóxica.

Palavras-chave: *Cecropia catarinenses*; efeito inibitório do crescimento; linhas celulares tumorais.

Abstract

Plant-derived natural products remain an important source of biologically active substances for cancer treatment. The evaluation of the genus *Cecropia* for cytotoxic activity is still poorly investigated, particularly the specie *Cecropia catharinensis,* whose study is reported here. This study reports the phytochemical composition of the leaves of *C. catharinensis* as well as the *in vitro* evaluation of cell growth inhibition by the crude methanolic extract (CME) and two semi-purified fractions (CME1 and CME2) against three human tumor cell lines: breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460) and melanoma (A375-C5). The crude methanolic extract CME was obtained by exhaustive extraction of the dried leaves of *C. catarinensis*. The less polar fraction CME1 was obtained from CME by its treatment with dichloromethane and ethyl acetate. The polar fraction CME2 corresponded to the insoluble residue obtained in the referred treatment. The effects of the crude extract and the two semi-purified fractions were evaluated on the growth of human tumor cell lines according to the procedure adopted by the National Cancer Institute (NCI, USA) in the "*In vitro* Anticancer Drug Discovery Screen". Results were expressed as GI_{50} ($\mu g.mL^{-1}$) values by means ± SD of at least three independent experiments performed in duplicate. CME inhibited the growth of the three human tumor cell lines used in this biological assay. The fraction CME1 was not only active against the panel of cancer cells but also presented the highest inhibitory activity among the three tested samples. The fraction CME2 showed moderate activity against MCF-7 and NCI-H460 cells and was inactive towards A375-C5 cells. These results indicate that *Cecropia catarinensis* can be considered as a potential source of cytotoxic compounds. To the best of our knowledge, there has been no report on the cytotoxic activity for *Cecropia catarinensis*.

Keywords: *Cecropia catarinensis*; growth inhibitory effect; tumor cell lines.

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The Cytotoxic Effect of Extracts Obtained from *Cecropia catharinensis* Cuatrec (Urticaceae)

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1. Introduction

Plants are known as a prevailing source of chemical compounds with important pharmacological activities. In the last 30 years, most of the newly discovered chemical entities from natural products were anticancer drugs being plants the major source of these compounds.[1,2]

The genus *Cecropia* includes about 60 species, which are widely distributed in Latin America, usually reach 5-15 feet tall, have leaves arranged in whorls. These plants has abundant seed production, which depends directly from sunlight for germination, rapid growth and short life cycle (25-50 years). These species has an important role in natural regeneration process of vegetation in areas that were completely deforested.[3]

The scientific interest on *Cecropia* has emerged from the traditional use of in folk medicine.[4] Within the genus *Cecropia*, for example the species *C. glaziovii*, *C.*

pachystachya and *C. catharinensis* were studied, regarding their chemical composition as well as and the biological evaluation of their extracts.[4]

Figure 1. Source: Eduardo L. H. Giehl – Digital flora from Rio Grande do Sul. (http://www.ufrgs.br/fitoecologia/florars/open_sp.php?img=570)

Previous studies confirmed the presence of secondary metabolites in genus *Cecropia* such as benzoic acid, cinnamic acid, flavonoids (kind flavone, flavonol, flavan-3-ol or cathechin and flavolignan) tannins, and triterpenes.[3]

Considering the main studies about plants belonging to the genus *Cecropia* they are pointed out as follows.

The triterpenoids euscaphic acid **(1)**, tormentic acid **(2)**, 2α-acetyl tormentic acid **(3)** and 3β-acetyl tormentic acid **(4)** were isolated from the roots of *C. lyratiloba* by countercurrent chromatography (Figure 2). All the four compounds were evaluated *in vitro* against sensitive (K562) and multidrug resistant (Lucena-1) leukemia cell lines. Lucena-1 cells derive from K562 but overexpress P-gp (glycoprotein-P).[5]

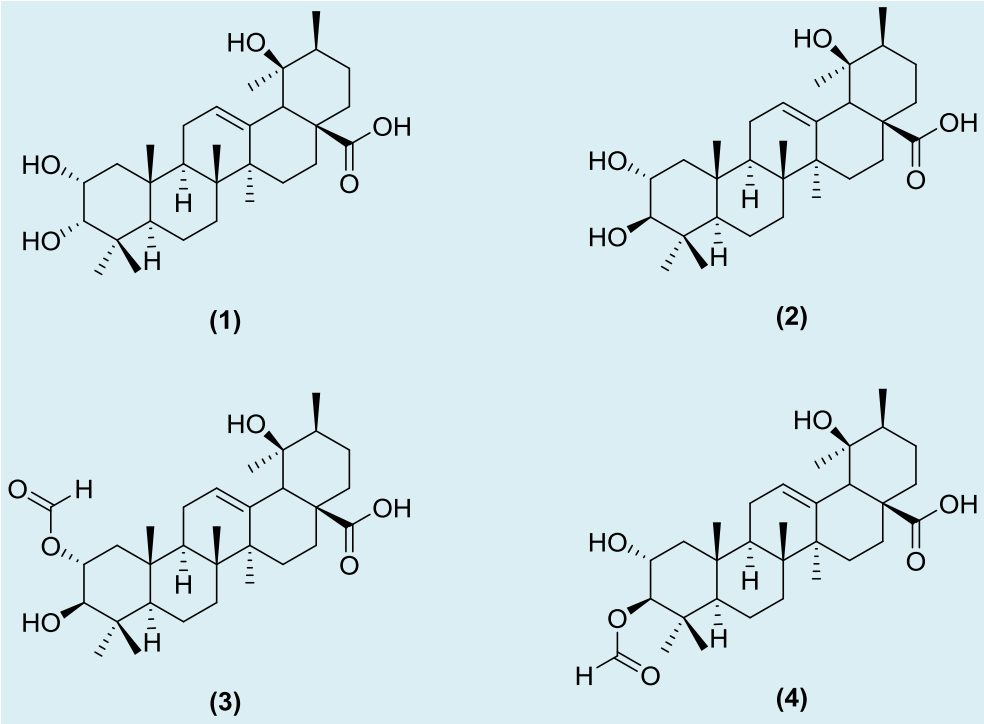

Figure 2. Triterpenoids isolated from the roots of *C. lyratiloba*

Those compounds were active to both K562 and Lucena-1 cell lines, with the following IC_{50} values: **(1)** 76.71 ± 8.23 µM towards K562 and 83.79 ± 4.17 µM against Lucena-1; **(2)** 89.36 ± 2.23 µM towards K562 and 80.25 ± 8.68 µM against Lucena-1; **(3)** 38.35 ± 5.29 µM towards K562 and 41.38 ± 4.16 µM against Lucena-1; **(4)** 56.61 ± 9.95 µM towards K562 and 72.87 ± 4.71 µM against Lucena-1. The structure/activity relationship (SAR) of tormentic acid referred, based on the obtained IC_{50} values, and indicated that the acetylation at C2 in the compound **(3)** increased the cytotoxic activities while acetylation at C3 in **(4)** did not show effect. The cell death mechanism of **(1)** was also assessed through the evaluation of the cytotoxic activities against human tumor cell lines, namely lung (A549 – IC_{50} = 95.58 ± 5.14 µM), colon (Caco-2 – IC_{50} = 04.1 ± 0.68 µM) and larynx (HEp-2 – IC_{50} = 105.7 ± 0.39 µM) cell lines, and by analyzing the cell cycle through the induction of apoptosis in K562 which indicated that **(1)** may induce apoptosis in a caspase-dependent way.[5] The mechanism of antineoplastic activity of **(4)** was also investigated against Lucena-1 cells. 3β-Acetyl tormentic acid **(4)** induced DNA fragmentation, activation of caspase-3 and cytochrome c release which indicated that the compound may induces apoptosis by the intrinsic apoptotic pathway. Additionally, the compound did not interfere with the expression nor modulates the activity of P-gp and was not a substrate to this transporter.[6]

The methanolic extract of *C. pachystachya* leaves reduced significantly the viability of Jurkat cells (human T cell lymphoblast-like cell line), HL60 cells (human promyelocytic leukemia cells) and HL60. Bcl-2 cell line (HL60 cells with ectopic expression of Bcl-2, known to be resistant to some anticancer drugs). The percentage of cell viability inhibition of the methanolic extract at the concentration of 20 $\mu g.mL^{-1}$ were 56.9 ± 11.6 to HL60 cells, 54.6 ± 2.5 to HL60.Bcl2 cells and 51.0 ± 14.3 to Jurkat cells.[7] The methanolic extract obtained from the leaves of *C. pachystachya* was also tested for cytotoxicity against four tumors cell lines and the results were IC_{50} > 125 $\mu g.mL^{-1}$ to B16 (murine melanoma cells), MCF-7 (human breast adenocarcinoma cells) and HCT-8 (human ileocecal adenocarcinoma

cells), and IC_{50} = 95 µg.mL^{-1} to HL-60 (human promyelocytic leukemia cells).[8]

In another study, the methanolic extract obtained from the leaves of *C. pachystachya* inhibited significantly glutathione S-transferase (GST) expression in L37 hepatocytes and Jurkat cells at 10 µg.mL^{-1}. The overexpression of GST in tumor cells is usually associated with resistance to chemotherapeutic drugs. *C. pachystachya* also exhibited cytotoxic activity against Jurkat cells at 100 µg.mL^{-1}.[9] The dichloromethane extract obtained from *C. pachystachya* and an isolated triterpenoid (pomolic acid) inhibited the viability of human polymorphonuclear (PMN) cells through apoptosis increasing the percentage of apoptotic PMN cells by 42% and 71% at 100 µM and 200 µM.[10]

There are still few studies focusing on the antitumor potential of the genus *Cecropia*. Our goals were to look for the *in vitro* growth inhibitory activity of the crude extracts of leaves of *C. catarinensis* in the following three human tumor cell lines: breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460) and melanoma (A375-C5). The results of this evaluation will allow the bioassay-guided fractionation of the most active samples in order to isolate the compounds that may responsible for the cytotoxic effects.

2. Material and Methods

General experimental

For analytical work, pre-coated silica gel plates, GF-254 (Macherey-Nagel, Germany), ALUGRAM®, and Sil G/UV254 (20x20 cm) were used and silica gel GF 254 (Merck, USA) was used for TLC (thin-layer chromatography). The used solvents were from Pronalab (Lisbon, Portugal), Carlos Erba (Milano, Italy) and Fisher Scientific (Waltam, USA) with 'pro-analysis' and 'pure' degree of purity. Solvents were evaporated at reduced pressure, using Buchi water bath B-480 and 461, Buchi rotavapor R-114 and RE-111, and Buchi B-169 vacuum-system, Switzerland.

Plant material

The leaves of *Cecropia catharinensis* were collected in Mendes (Rio de Janeiro, Brazil) 2001, data GPS: 22°31'42.09''S/43°44'21.31''O, by Dr. Douglas Siqueira de Almeida Chaves from the Universidade Federal Rural do Rio de Janeiro (Brazil) and were identified by Professor Jorge Pedro Carauta (Museu Nacional – JB/UFRJ). A voucher specimen was deposited in the Museu Nacional do Rio de Janeiro (Brazil) under the number R212.173.

Crude extract and fractions

The crude methanolic extract (CME) was obtained using 1 kg of the dried leaves of *C. catharinensis* by exhaustive extraction with methanol (5L) at room temperature. CME (150.29 g) was purified by extraction with different solvents with increasing polarity. CME was treated with dichloromethane (1x10 L; CH_2Cl_2) and extracted at room temperature. The fraction, soluble in CH_2Cl_2, was then concentrated at reduced pressure and named F1 (11.60 g). The CH_2Cl_2 insoluble residue was treated with ethyl acetate (1x11 L; AcOEt) and the soluble fraction, that resulted from the extraction with AcOEt, was also concentrated at reduced pressure and named F2 (28.13 g). Fractions F1 and F2 were analyzed by TLC (Sil Gel, $CHCl_3$ – MeOH 95:5) and were combined to give CME1 (38.20 g). The insoluble residue resulting from the extraction with dichloromethane and ethyl acetate was finally concentrated at reduced pressure and named CME2 (109.75 g).

Cell cultures

Three human tumor cell lines, MCF-7 (ECACC, breast adenocarcinoma, UK), NCI-H460 (an offer from NCI, Bethesda, USA, non-

small cell lung cancer), and A375-C5 (ECACC, melanoma, UK) were used. Cells were routinely maintained in RPMI-1640 medium (RPMI-1640, BioWhitakker®, Lonza, USA), supplemented with 5% inactivated FBS (Gibco, Spain), at 37°C in a humidified atmosphere containing 5% CO_2. Cell number and viability were routinely determined with Trypan blue exclusion assay (Sigma-Aldrich, USA). All the experiments were performed with cells in exponential growth and presenting more than 90% viability.

Growth inhibition of human tumor cell lines

The effect of crude extract (CME) of the *C. catarinensis*, CME1 and CME2 were evaluated on the growth of human tumor cell lines according to the procedure adopted by the National Cancer Institute (NCI, USA) in the "*In vitro* Anticancer Drug Discovery Screen"[11] which uses the protein-binding dye sulforhodamine B (SRB) (Sigma-Aldrich, USA), to assess cell growth. [12] This colorimetric assay indirectly estimates cell number by staining cellular protein with the protein-binding dye SRB. Each cell line was plated at an appropriate density (MCF-7 and NCI-Bio, H460 at 5 x 10^3 cells/well, A375-C5 at 7.5 x 10^3 cells/well) in 96-well plates and allowed to attach for 24 hours.[13] Exponential growing cells were further exposed for 48 hours to five serial concentrations of CME, CME1 and CME2. Following this incubation period, the adherent cells were fixed with 10% trichloroacetic acid (Sigma-Aldrich, USA) (final concentration), washed with 1% acetic acid and stained with SRB. The bound stain was solubilized with 10 mM Tris and the absorbance was measured at 492 nm in a microplate reader (Model 680 Microplate Reader model, Bio-Rad). The concentration that inhibited cell growth in 50% (GI_{50}) was calculated as previously described.[14] The effect of the vehicle solvent (DMSO, Sigma-Aldrich, USA) on the growth of these cell lines was also determined by exposing untreated control cells to the maximum concentration of DMSO used in each assay (0.25%).

3. Results and Discussion

In this study, we evaluated the *in vitro* growth inhibitory activity of the crude extract (CME) and two subfractions (CME1 and CME2) of *C. catharinensis* on three human tumor cell lines: breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460) and melanoma (A375-C5), by the SRB assay (table 1).

Table 1. Growth inhibitory (GI_{50}) of fractions CME, CME1 and CME2 in MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) cell lines

	GI_{50} (µg.mL^{-1})		
Extracts	MCF-7 (breast adenocarcinoma)	NCI-H460 (non-small cell lung cancer)	A375-C5 (melanoma)
CME	58.8 ± 9.1	68.3 ± 7.2	80.2 ± 4.9
CME1	31.8 ± 6.7	36.1 ± 9.8	42.1 ± 8.8
CME2	80.9 ± 19.2	85.4 ± 27.2	≥ 200
Doxorubicin	60.3 ± 1.2*	19.6 ± 1.9 *	130.0 ± 25.2*

* Results expressed in µM.

Results are expressed as GI_{50} ($\mu g.mL^{-1}$) values of the extracts tested using MCF-7, NCI- H460 and A375-C5 cell lines by means ± SD of at least three independent experiments performed in duplicate. Doxorubicin was used as a positive control.

After incubation for 48 hours, the results showed that the crude extract CME inhibited the growth of the three tumor cell lines. In order to further identify the compounds responsible for the growth inhibitory activity, the CME was semi-purified in a less polar fraction CME1 and a polar fraction CME2. Our preliminary results demonstrated that the non-polar fraction CME1 was active against the three cell lines used in the assay. In turn, CME2 was active towards NCI-H460 and MCF-7 cells but it was inactive against A375-C5 cells.

The CME1 presented the highest growth inhibitory activities for the three tumor cell lines. These results suggest that the more active compounds are in the less polar fraction (CME1) or this fraction may be more concentrated in active compounds. However, the CME2 showed the lowest inhibitory activities for breast and lung cancer cell lines, being inactive for melanoma cells. This finding may enhance the idea that the active compounds are less polar.

The overall results indicated that the crude extract obtained from the leaves of *C. catharinensis* may contain compounds with cytotoxic activity. According to the chemical study of Machado *et al*., that reported the isolation of eleven pentacyclic triterpenes from the roots and stems of *C. catharinensis*[15], and the studies of cytotoxicity to triterpenoids from *C. lyratiloba*[5,6] and *C. pachystachya*[10], we can suggest that some of the active compounds presented in the extracts of this study could be structurally triterpenoids. Indeed, the comparative analytical TLC of the three extracts (CME, CME1 and CME2) showed that there were two violet /red spots in the plate (80% of H_2SO_4 in methanol) and these spots (Rf= 0.76 and 0.73; eluent: CH_2Cl_2 + acetone 9:1) were more pronounced with the less polar fraction CME1. In accordance to this bioassay-guided evaluation, further chemical and biological studies of CME1 are ongoing.

To the best of our knowledge, this is the first report for the growth inhibitory activity of tumor cell of *Cecropia catarinensis* towards NCI-H460, MCF-7 and A375-C5 tumor cell lines.

4. Conclusions

The crude extract CME and semi-purified fractions (CME1 and CME2) were investigated for their capacity to inhibit the *in vitro* growth of three tumor cell lines: MCF-7, NCI-H460 and A375-C5. Results showed that CME was active against the referred panel of cancer cell lines. Interestingly, the less polar fraction (CME1) presented the highest inhibitory activity for the same. Further investigations to exploit the potential of this species on antitumor therapy are needed.

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