

Cytotoxic evaluation of essential oil from *Zanthoxylum rhoifolium* Lam. leaves

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ABSTRACT

Zanthoxylum rhoifolium Lam is a plant popularly used as antimicrobial, for malaria and inflammatory treatment. The essential oil of *Z. rhoifolium* was extracted and its cytotoxic effects against HeLa (human cervical carcinoma), A-549 (human lung carcinoma), HT-29 (human colon adenocarcinoma), Vero (monkey kidney) cell lines and mice macrophages were evaluated. Some of the terpenes of its essential oil (β -caryophyllene, α -humulene, α -pinene, myrcene and linalool) were also tested to verify their possible influence in the oil cytotoxic activity. The results obtained permitted to confirm that the essential oil is cytotoxic against tumoral cells (CD_{50} = 82.3, 90.7 and 113.6 μ g/ml for A-549, HeLa e HT-29 cell lines, respectively), while it did not show cytotoxicity against non-tumoral cells (Vero and mice macrophages). Thus, the essential oil from *Z. rhoifolium* leaves seems to present a possible therapeutic role due to its selective cytotoxic activity against tumoral cell lines.

KEYWORDS

Zanthoxylum, Essential oil, cytotoxicity, β -caryophyllene, α -humulene

Avaliação citotóxica do óleo volátil extraído das folhas do *Zanthoxylum rhoifolium* Lam.

RESUMO

O *Zanthoxylum rhoifolium* Lam. é uma planta popularmente utilizada como antimicrobianos, no tratamento da malária e de inflamações. O óleo volátil do *Z. rhoifolium* foi extraído e posteriormente foi avaliada a sua citotoxicidade contra células HeLa (carcinoma cervical humano), A-549 (carcinoma de pulmão humano), HT-29 (adenocarcinoma de cólon humano), Vero (rim de macaco) e macrófagos de camundongos. Alguns terpenos constituintes do óleo volátil (β -cariofileno, α -humuleno, α -pineno, mirceno e linalool) também foram testados para verificar as possíveis influências sobre a citotoxicidade do óleo. Os resultados obtidos permitiram verificar que o óleo volátil é citotóxico contra células as tumorais (CD_{50} = 82.3, 90.7 e 113.6 μ g/ml para A-549, HeLa e HT-29 cell lines, respectivamente), mas não apresenta citotoxicidade contra as células não tumorais (Vero e macrófagos de camundongos). Desta forma o óleo volátil das folhas do *Z. rhoifolium* demonstra possuir uma possível ação terapêutica em função de sua citotoxicidade contra células tumorais.

PALAVRAS-CHAVE

Zanthoxylum, Óleo Volátil, Citotoxicidade, β -cariofileno, α -humuleno

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INTRODUCTION

Zanthoxylum rhoifolium Lam. is a plant from South America popularly employed against inflammatory, microbial and malaria processes. Currently, *Z. rhoifolium* has been commercialized as a component of weed mixtures in teas and infusions sold in drugstores, supermarkets and popular markets (Gonzaga *et al.*, 2003; Da Silva *et al.*, 2006).

Zanthoxylum genus, particularly the species *Z. rhoifolium*, is found in tropical and temperate regions in the world and in Brazil it is found in all regions, including the Amazon forest (Floyd, 1989; Lorenzi, 1992). The wood from trees of *Zanthoxylum* genus is employed in the Amazon region for agricultural instruments manufacturing, also oars, bodyworks and civil construction (Le Cointe, 1947; Cowan & Smith, 1973; Lorenzi, 1992; Loureiro & Lisboa, 1992).

Recently, studies of chemical composition of essential oil from *Zanthoxylum ekmanii* leaves collected in the Amazon forest determined that the compounds germacrene D and β -caryophyllene are the major constituents in the essential oil of this specimen (Facundo *et al.*, 2003). As much as *Z. Rhoifolium*, the *Z. Ekmanii* is utilized by the inhabitants that live along the Madeira River (Porto Velho – Rondônia) for cancer and malaria treatment, teeth pain relief and for reduce microbial processes (Gonzaga *et al.*, 2003; Facundo *et al.*, 2005).

This genus embraces species used for different medical purposes, and some attention has been given to the toxic effects of its different species. Islam *et al.* (2001) verified that etheric extracts from the bark of *Z. rhesta* are toxic to *Artemia salina* which, on the other hand, does not suffer toxic action from chlorophormic extracts of *Z. budrunga*; Ju *et al.* (2001) showed that *Z. americanum* presented substances, as dipetaline, alloxanthoxyletin e sesamin, that inhibit the DNA synthesis in HL-60 cells; Rodrigues *et al.* (1998) reported the hexane extracts of *Z. naranjillo* induced alterations in albumin and alkaline phosphatase levels in mice, without liver or biliary alterations, which suggests a possible absence of clinical toxicity. From these studies, it is possible to confirm several potential therapeutical uses of plants from the genus *Zanthoxylum*.

In recent times, the composition of the essential oil of *Zanthoxylum rhoifolium* Lam. leaves was determined (Moura *et al.*, 2006). Despite not being preponderant, the oil analyses showed that among its numerous constituents, there are some components described in literature as cytotoxic substances against tumoral cells, such as β -caryophyllene (5.9%), β -elemene (3.1%), δ -elemene (1.6%), α -humulene (2.3%) (Duh *et al.*, 1999; Legault *et al.*, 2003; Sibanda *et al.*, 2004; Sylvestre *et al.*, 2005; Stravi *et al.*, 2005; Tatman and Mo, 2002; Wang *et al.*, 2005; Sylvestre *et al.*, 2006; Hou *et al.*, 2006; Tao *et al.*, 2006; Xiao *et al.*, 2006. Some other components with no or low antitumoral activity were also found in this species, eg

sphathulenol (3.8%), *d*-pinene (6.4%), linalool (0.5%), myrcene (0.6%) (Fullas *et al.*, 1994; Tatman & Mo, 2002; Stavri *et al.*, 2005).

In this work, the essential oil extracted from the leaves of *Zanthoxylum rhoifolium* Lam. was submitted to antitumoral evaluation as well as some of its constituents (β -caryophyllene, α -humulene, α -pinene, myrcene and linalool) against HeLa, A-549, HT-29, Vero cell lines and mice macrophages).

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

Fresh *Z. rhoifolium* leaves were collected in the city of Chapecó, SC, Brazil, in December 2005. The species were identified by botanists from the Federal University of Santa Maria (Santa Maria, RS, Brazil) herbarium, where a voucher was deposited under the number HDFI-265.

EXTRACTION OF THE ESSENTIAL OIL

The essential oil was obtained from 1000 g of leaves that were submitted to hydrodistillation for 4 h in a modified Clevenger device, resulting in a light yellow oil (yield of 0.84%). The oil was dried with anhydrous sodium sulphate and stored at -20° C (Da Silva *et al.*, 2006; Moura *et al.*, 2006).

CHEMICAL COMPOUNDS

The sesquiterpene hydrocarbons (β -caryophyllene and α -humulene), monoterpene hydrocarbons (α -pinene and myrcene) and oxygenated monoterpene (linalool) were purchased from 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 were grown in RPMI 1640 supplemented with 10% fetal calf serum 1% (w/v) glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 5 μ g/ml amphotericin B. Cells were cultured in a humidified atmosphere at 37 °C in 5% CO₂. The macrophages were isolated from mice and kept according to the methodology described by Tseng *et al.* (2006).

IN VITRO ASSAY CYTOTOXIC ACTIVITY

Cells were washed with phosphate-buffered saline (PBS) free of magnesium and calcium. After PBS decantation, 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 with a medium to yield previously determined optimal plating densities for cells.

Before the assay, 5×10^4 cells / well were seeded on 96-well plates and the suspension was incubated 24 h at 37°C to cell attachment. After 24 h, the cells were treated with the essential oil and terpenes. 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 against all cell lines and macrophages. Terpenes were also dissolved in ethanol and tested to five replicate wells, but over the range of 200 - 0.2 µg/ml. 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 terpenes were considered according to the survival of 50% or less cells after an exposure time of 72 h. The cell culture used as control received only 0.5 % ethanol at final concentration.

Cytotoxicity was measured using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After an exposure time of 72 h, 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) in PBS was incubated with cells for 2 h at 37°C. After this period, the medium containing MTT was removed and 100 ml of acidified isopropanol (0.04 mol/l HCl) was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, model 3550, USA). Cell viability was expressed with respect to the absorbance of the control wells, which were considered as 100% of absorbance. Cytotoxicity is expressed as the concentration of the substance (essential oil and terpenes) 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).

STATISTICAL ANALYSIS

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

RESULTS

The cytotoxic assays results obtained with the essential oil extracted from the leaves of *Z. rhoifolium* Lam. and with monoterpene hydrocarbons (α -pinene and myrcene), oxygenated monoterpene (linalool) and sesquiterpene hydrocarbons (β -caryophyllene and α -humulene) against HeLa (human cervical carcinoma), A-549 (human lung carcinoma), HT-29 (human colon adenocarcinoma), Vero (monkey kidney) cell lines and mice macrophages are resumed in Table 1.

The components of the essential oil from *Z. rhoifolium*, myrcene and linalool, up to a concentration of 200 µg/ml, did

not present cytotoxic activity up to a concentration of 200 µg/ml against any of the cells tested. Another component tested, α -pinene up to 200 µg/ml concentration, was also not cytotoxic to Vero and HT-29 cells and mice macrophages, but presented some degree of cytotoxic activity against A-549 and HeLa cell lines (CD₅₀ values were 183.2 and 172.7 µg/ml, respectively).

β -caryophyllene and α -humulene (sesquiterpene hydrocarbons) showed a much higher cytotoxicity against A-549, HeLa and HT-29 cell lines when compared to CD₅₀ values of *Z. rhoifolium* essential oil (see Table 1). Vero cells were susceptible to both sesquiterpenes, but CD₅₀ values were much higher than those obtained by α -caryophyllene and α -humulene against A-549, HeLa e HT-29 cell lines (CD₅₀ values 80.3 and 109.7 µg/ml, respectively). Mice macrophages were sensitive only to α -humulene, whose CD₅₀ value (179.3 µg/ml) was also higher than those obtained by the other cell lines tested.

DISCUSSION

Studies have shown differential sensitivities to several natural compounds between tumor and normal cells in vitro or in vivo, and the results obtained from the present study show that the essential oil from *Zanthoxylum rhoifolium* Lam. is cytotoxic to A-549, HeLa and HT-29 tumoral cell lines. Nevertheless, it is not cytotoxic to others non-tumoral cells tested (Vero cell and mice macrophages) (Shoff *et al.*, 1991; Elson, 1995; Yu *et al.*, 1995; He *et al.*, 1997; Burke *et al.*, 1997). In fact, it is interesting to notice, from the results in Table 1, that non-tumoral cells (macrophages and Vero cells) were almost insensitive to the studied compounds and to the essential oil, which thus exhibits some selectivity degree against tumor cells.

The composition analysis of the essential oil from *Z. rhoifolium* showed the presence of several terpenes, described in the literature, against many cell lineages. Nonetheless, we believe that the most accepted hypothesis for explaining the toxic effects verified in this study against A-549, HeLa and HT-29 cell lines might not be attributed to any of its specific constituents, but to the

Table 1 – Cytotoxic activity of essential oil from leaves of *Z. rhoifolium* in mammalian cells lines and some components (CD₅₀ - µg.ml)^a

	Vero	Macrophages	A-549	HeLa	HT-29
Essential oil	> 600 ^b	> 600	82.3 ± 11.1	90.7 ± 8.2	113.6 ± 4.4
β -caryophyllene	80.3 ± 8.3	> 200	21.3 ± 0.4	19.8 ± 0.6	32.8 ± 0.5
α -humulene	109.7 ± 2.3	179.3 ± 3.6	18.7 ± 0.9	29.0 ± 0.3	21.8 ± 0.4
α -pinene	> 200	> 200	183.2 ± 11.1	172.7 ± 8.2	> 200
myrcene	> 200	> 200	> 200	> 200	> 200
linalool	> 200	> 200	> 200	> 200	> 200

^a Average of three independent determinations; five replicates; values are mean ± SEM.
^b Viable cells in all the concentrations tested.

synergism between the cytotoxic effects of some components of the essential oil from the plant.

Studies showed that some compounds, such as myrcene, linalool and α -pinene, were completely inactive or presented low toxicity against the cells A-549, HeLa and HT-29 in the concentration range utilized. Some other tested compounds, such as β -caryophyllene and α -humulene, showed high cytotoxic activity against these same cells. (see Table 1).

In fact, Sylvestre *et al.* (2005) showed that myrcene is a low cytotoxic compound against DLD-1 cells. Sibanda *et al.* (2004) demonstrated that linalool is a compound with some antimicrobial activity, but not cytotoxic against SK-MEL-28, MDA-MB-231, MCF7, PC-3 and Hs 578T cells. However, Tatman & Mo (2002) verified that both linalool and α -pinene present cytotoxic activity against murine B16 melanoma and human HL-60 leukemia cells. α -humulene e β -caryophyllene were described as cytotoxic compounds against A-549, DLD-1, M4BEU, HeLa, Bel-7402 and CT-26 cells (Legault *et al.*, 2003; Sylvestre *et al.*, 2005; Sylvestre *et al.*, 2006; Hou *et al.*, 2006).

Thus, it is possible to observe that the essential oil from leaves of *Z. rhoifolium* presents an interesting biological activity concerning to its selective cytotoxic activity against A-549, HeLa and HT-29 tumoral cell lines, while it is not cytotoxic to Vero cell and mice macrophages (non-tumoral cells). Some other studies involving different cell lines and animal models might characterize the presence of other substances potentially active against several types of tumors.

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