

The anticancer activity of dichloromethane crude extract obtained from *Calea pinnatifida*

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Abstract: The genus *Calea* is reported for many biological activities such as antiinflammatory, antiplasmodial, antifungal, antimicrobial, and cytotoxic activities. Most of the pharmacological activities are credited to germacranolides, a sesquiterpene lactone common to this genus. Dried aerial parts of *Calea pinnatifida* Banks were extracted with dichloromethane, which generated the dichloromethane crude extract (DCE). The main purpose of this study was to evaluate the anticancer activity of DCE performed in sulforhodamine B cytotoxicity in vitro assay against human cancer cell lines and in vivo Ehrlich models. The DCE showed a high potency and selectivity for the melanoma and kidney cell line. Two in vivo assays were also conducted in the Ehrlich ascites tumor and Ehrlich solid tumor. In the Ehrlich ascites tumor assay, the treatment with DCE increased survival rates at the highest dose (200 mg/kg). Interestingly, in the Ehrlich solid tumor, by increasing the number of treatments from one to three times a week, the tumor growth was inhibited by a lower dose (100 mg/kg). These results encouraged follow-up studies with *C. pinnatifida* in order to identify the active principles and to determine the anticancer mechanism of action.

Keywords: *Calea pinnatifida*, cytotoxicity, cancer, Ehrlich ascitic tumor, Ehrlich solid tumor

Introduction

Plants have served as a rich source of therapeutic agents for many centuries, being used themselves or as the basis for synthetic drugs. Despite the great developments in organic synthesis, 55% of recent chemotherapeutic drugs are derived from or based upon natural products.¹ The use of plants as food and in folk and traditional medicine has led to their being one of the main agents in the research and development of cancer chemopreventive drugs.^{2,3}

Calea pinnatifida Banks is used in folk medicine in the treatment of stomach ache, giardiasis, and amoebiasis.⁴ Belonging to the Asteraceae family, extracts from the leaves of this plant are composed chiefly of fatty esters, 4-glycosyloxybenzoic acid, anisic acid, sitosterol, stigmasterol, polyacetilene, and germacranolides.⁵

The genus *Calea* has approximately 110 species. It is found in tropical and subtropical regions, and contains germacranolides as a common chemical component.⁴⁻⁶ Many biological activities, including antiinflammatory, antiplasmodial, antileishmanial, acaricidal, antifungal, antimicrobial, and cytotoxic, have been reported for this genus.⁶⁻¹² Germacranolides have also been reported to have other properties such as the ability to induce apoptosis, cytotoxicity, antifungal activity, and inhibitory activity against NF- κ B.^{6,10-14}

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Arucanolide, a germacranolide isolated from *C. urticifolia*, induced apoptosis in HL60 cells by dissipating the mitochondrial membrane potential, and triggering apoptosis-inducing factor.¹⁵ Among the compounds identified in *C. pinnatifida*, the germacranolides seem to be the most relevant substances, as they promote many biological activities in the potential development of anticancer agents.^{10–14}

Since previous literature has not examined *C. pinnatifida* and its anticancer-related influences, the aim of the present study is to examine the antitumor activity of the crude extract taken from this species in both in vitro and in vivo assays.

Materials and methods

Plant material

The aerial components of *C. pinnatifida* Banks were collected in wildcrafting and identified at the Multidisciplinary Center for Chemical, Biological, and Agricultural Research (CPQBA, University of Campinas, São Paulo, Brazil). A voucher specimen, which was positively identified by MSc Katia Calago Althoff, botanist, was deposited at the CPQBA Herbarium (Number 107).

Obtaining dichloromethane and ethanolic crude extract (DCE and ECE)

Dried aerial parts of *C. pinnatifida* were ground prior to use in a Stephen mill (model UM 40), and an aliquot (300 g) was extracted by soxhlet (1000 mL; 2 × 48 hours) with dichloromethane (Merck® KGaA, Darmstadt, Germany). This provided the dichloromethane crude extract (DCE) after solvent evaporation. The plant residue was extracted with ethanol 96°GL (Merck®) in the same conditions cited above. The solvent was evaporated at 45°C under vacuum, and it was subsequently lyophilized, resulting in ethanolic crude extract (ECE).

In vitro anticancer activity assay

Human tumor cell lines, UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (resistant ovary), 786-O (kidney), NCI-H460 (lung, non-small cell), PC-3 (prostate), OVCAR-3 (ovary), HT29 (colon), and K-562 (leukemia), were kindly provided by the US National Cancer Institute. Stock cultures were grown in medium containing 5 mL RPMI 1640 (Gibco®–BRL; Life Technologies, Carlsbad, CA) supplemented with 5% fetal bovine serum. Gentamicine (50 µg/mL) was added to the experimental cultures. Cells in 96-well plates (plates: 100 µL cells/well; inoculation density ranging from 4 to 7 × 10⁴ cells/mL) were exposed to sample concentrations in DMSO/RPMI (0.25, 2.5, 25, and 250 µg/mL) at 37°C, with

5% CO₂ in air for 48 hours. The final DMSO concentration did not affect cell viability. Following this preparation, cells were fixed with 50% trichloroacetic acid, and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay.^{16,17} Doxorubicin (doxorubicin chloridrate, 50 mg; 11% purity; Eurofarma, São Paulo, Brazil) was adopted as a positive control. Using the concentration response curve for each cell line, total growth inhibition (TGI) was determined through nonlinear regression analysis (Table 1) using ORIGIN 8.0 software (OriginLab Corporation, Northampton, MA).^{18–20}

In vivo assay – acute toxicity

BALB/c mice were treated intraperitoneally (IP) with DCE at doses of 100, 300, 500, and 1000 mg/kg. Groups were observed during 4 hours and then daily for 14 days. The following general toxicity parameters were evaluated: body weight loss, locomotion, behavior (agitation, lethargy), respiration, salivation, tearing eyes, cyanosis, and mortality.^{21,22} The lethal dose was calculated by linear regression, as described by Litchfield and Wilcoxon.²²

In vivo assay – Ehrlich tumor

DCE was evaluated in vivo on an Ehrlich ascitic tumor (EAT) and Ehrlich solid tumor (EAT) assay. Ehrlich tumor cells were maintained in the ascites form by peritoneal passages in mice via weekly transplantation of 5 × 10⁵ tumor cells. Male BALB/c mice aged 8–10 weeks and weighing 25–30 g were used for Ehrlich tumor experiments. The animals were obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science-UNICAMP, and were maintained under controlled temperature conditions (22°C–24°C), light (12 hours of light, 12 hours of dark) and humidity (45%–65%), with

Table 1 Comparative TGI (µg/mL) in human tumor cell lines

Cell lines	Doxorubicin	DCE	ECE
UACC-62	0.04	13.77	>250
MCF-7	0.69	204.85	>250
NCI-ADR/RES	5.53	228.58	>250
786-O	0.79	8.41	>250
NCI-H460	0.05	44.22	>250
PC-3	0.87	24.35	>250
OVCAR-3	4.32	28.08	>250
HT-29	0.02	50.45	80.0
K-562	2.45	>250	>250

Abbreviations: TGI, total growth inhibition; DCE, dichloromethane crude extract; ECE, ethanolic crude extract.

food and water ad libitum. All procedures were conducted in accordance with the principles and guidelines adopted by the Institutional Committee for Ethics in Animal Research at the University of Campinas (CEEA, UNICAMP, protocol 1421-1, December 19, 2007).

Ehrlich ascites assay

Ehrlich tumor cells (1.0×10^4) were implanted IP, and the animals ($n = 10$) were treated with the samples, IP, once a week over the course of 21 days; the animals were followed for a total of 90 days. The animals were divided into three groups: negative control group (vehicle); positive control group (doxorubicin chloridrate, 50 mg; 11% purity) at a dose of 3.0 mg/kg; and DCE treated groups. DCE was administered at doses of 50, 100, and 200 mg/kg. Anticancer activity was assessed by the survival rates of the groups.^{3,23}

Ehrlich solid assay

Ehrlich tumor cells (1.0×10^5) were implanted subcutaneously in the region of the back, and the animals ($n = 8$) were treated with the samples, IP, every 48 hours over the course of 15 days. The animals were divided into groups: negative control group (vehicle); positive control group (doxorubicin chloridrate, 50 mg; 11% purity) at a dose of 3.0 mg/kg; and DCE treated groups. DCE was administered at doses of 50 and 100 mg/kg. The tumor volume was measured using a pachymeter (height \times width \times length). Anticancer activity was assessed by comparing the relative tumor weight (tumor weight/animal weight) and tumor volume using the equation:

$$\frac{(\text{Mean [Relative Tumor Weight or Volume] of the Negative Control Group} - \text{Mean [Relative Tumor Weight or Volume] of Treatment Groups}) / \text{Mean (Relative Tumor Weight or Volume) of the Negative Control Group} \times 100.}{3} \quad (1)$$

Statistical analyses

Results are expressed as the mean \pm standard deviation (SD) per group. Statistical evaluation was done using analysis of variance, followed by Duncan's new multiple range test using StatSoft® software (StatSoft, Inc, Tulsa, OK). Graphics were designed using the ORIGIN® software. Differences were considered significant at $P \leq 0.05$ and are represented by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

The evaluation of DCE in the in vitro sulforhodamine B assay demonstrated that this extract has a strong relationship

between its concentration and the effect on and selectivity for the melanoma (UACC-62) and kidney (786-O) cell lines.

The TGI shown in Table 1 demonstrated the activity of DCE, ECE, and doxorubicin. DCE is very potent and kills all cell lines; meanwhile, the ECE did not show any antiproliferative effects. Doxorubicin shows high cytotoxic activity in almost all cell lines except for NCI-ADR/RES, which expresses a multidrug resistance phenotype.

Subsequently, the DCE was selected for in vivo studies. Through IP administration, DCE showed a 50% lethal dose of 500 mg/kg in an acute toxicity test. The IP route was chosen because the absorption barriers are similar to the intravenous route, which more closely resembles current clinical applications for chemotherapy treatment. The results of this toxicity study established the doses used for in vivo assays.²²

The in vivo EAT anticancer activity was determined by the survival life span by comparing the mortality rate of the treatment groups versus the control group (Figure 1). Only the highest dose (200 mg/kg) increased the survival rate when compared with the negative control group ($P = 0.009$). In spite of the antitumor activity, the 200 mg/kg dose caused the death of one experimental animal before the negative control group; therefore, the dose had no relationship with tumor development. Both the 50 mg/kg and the 100 mg/kg doses did not show any anticancer activity in this assay using once a week treatment, and the positive control (doxorubicin) increased the overall survival time, demonstrating antitumor activity, as expected ($P < 0.001$).

In vivo EST anticancer activity was determined by the reduction of the relative tumor weight and tumor volume. In this assay, 100 mg/kg of DCE showed anticancer activity in a treatment that was administered every 48 hours. This dose inhibited 66.7% of the relative tumor weight ($P = 0.0002$)

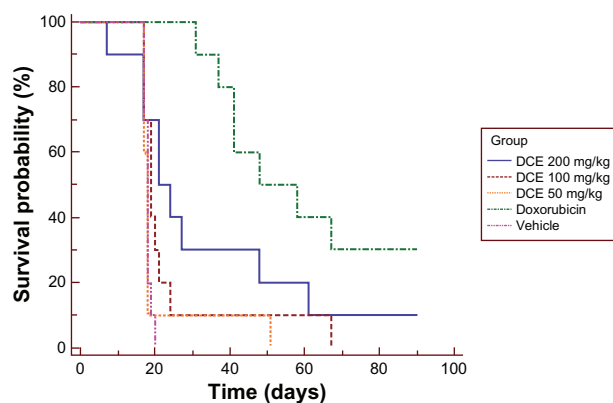


Figure 1 Kaplan-Meier survival curves of mice treated with *Calea pinnatifida* DCE. **Notes:** Doses of 50 mg/kg ($P = 0.973$), 100 mg/kg ($P = 0.202$), and 200 mg/kg ($P = 0.009$). Controls: vehicle (negative) and doxorubicin (positive) ($P < 0.001$). **Abbreviation:** DCE, dichloromethane crude extract.

and 57.6% of tumor volume ($P = 0.012$), whereas the 50 mg/kg dose increased the tumor weight ($P = 0.004$), but did not show any differences in the volume from negative control ($P = 0.06$). The positive control (doxorubicin) inhibited 71.6% of the tumor weight ($P = 0.0007$) and 66.5% of tumor volume ($P = 0.032$) (Figure 2).

Discussion

Because of the high number of cancer cases, the interest in alternative therapies using natural products are increasing, especially those derived from plants.^{24,25} In order to look for new sources of therapeutic anticancer agents, many plant extracts and active principles have been studied in in vitro and in vivo cancer models, and the correlation of both studies became one of the key steps for the success of this type of research.¹

Since the knowledge that different cell lines display varying sensitivities towards cytotoxic compounds, the use of more than one cell line is considered necessary for the detection of anticancer compounds.²¹ Bearing this in mind, cell lines of different histological origins were used in the present study. An in vivo model could potentially broaden these results, but more data needs to be compiled prior to this being a viable option to overpass the limitations of the in vitro models.²⁶

Previous studies demonstrated that other *Calea* species have many different biological properties, such as cytotoxicity and antiinflammatory activities.^{6,7} However, the present study showed, for the first time, the antitumor activity of *C. pinnatifida* crude extract, both in in vitro and in vivo experimental models. The DCE inhibited cell growth at low concentrations ranging from 8.0 to 70.0 $\mu\text{g/mL}$

(TGI value) with highest selectivity noted for kidney (786-O) and melanoma (UACC-62) cell lines with TGI of 8.41 and 13.77 $\mu\text{g/mL}$, respectively (Table 1). These data were used as the determining point to study the activity of DCE in a murine cancer model.

In the EAT model, the *C. pinnatifida* DCE antitumoral activity was confirmed by the prolongation of the life-span in once-a-week treated groups at the dose of 200 mg/kg. These results are important since the EAT is a very aggressive tumor,²⁷ killing the negative control group after 20 days of tumor cells inoculation. The EAT implantation induced a local inflammatory reaction, increasing vascular permeability and resulting in cellular migration and progressive ascites fluid formation.²³ The ascites fluid formation is essential for tumor growth, as this constitutes the direct nutritional source for tumor cells.^{3,28} Since the extract increased animal survival, these findings suggest either a direct cytotoxic effect on tumor cells, as previously observed in vitro, or an indirect local effect provided by an antiinflammatory activity as described by other species of the genus *Calea*.^{3,5,29}

To investigate whether the inhibitory effect of *C. pinnatifida* on Ehrlich tumors would be local or systemic, we evaluated the effect of IP treatment in another cancer model, the EST. In order to avoid the acute toxicity observed in the EAT model at a dose of 200 mg/kg, we increased the frequency of dosing and reduced the maximum dose administered. The smaller interval between treatments proved to yield better anticancer activity since 100 mg/kg was able to diminish the tumor size in EST, whereas the same dose did not increase the survival rate in the EAT model. The possible explanation for these results may be the maintenance

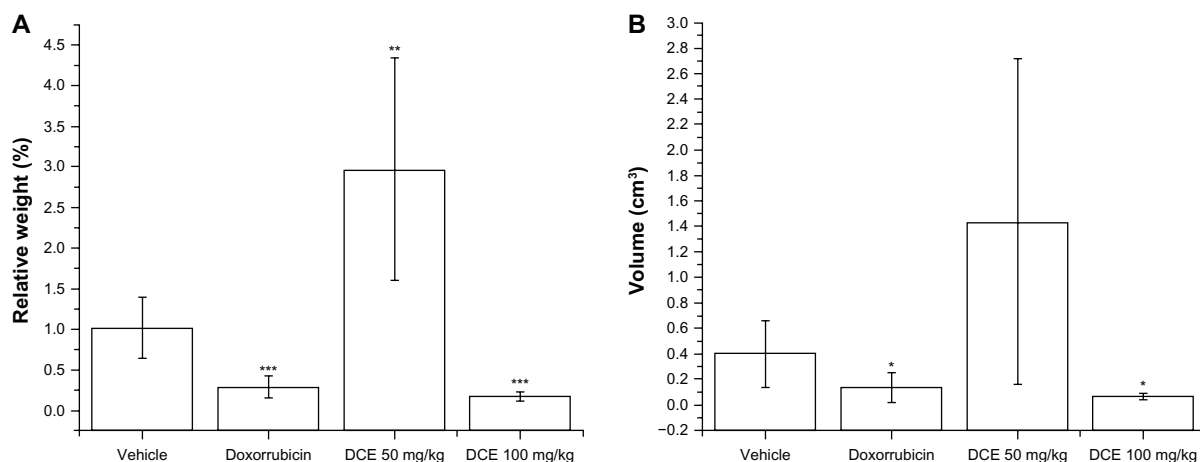


Figure 2 Ehrlich solid relative tumor weight and tumor volume of mice treated with *Calea pinnatifida* DCE. Ehrlich solid relative tumor weight (A) and tumor volume (B) of mice treated with *C. pinnatifida* DCE.

Notes: Doses of 50 mg/kg and 100 mg/kg. Controls: vehicle (negative) and doxorubicin (positive). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Abbreviation: DCE, dichloromethane crude extract.

of DCE blood levels in an every-two-day schedule rather than a once-a-week treatment schedule, when using the same dose level. However, further pharmacokinetics studies are required to determine the half-life of DCE.

Despite the anticancer activity of the DCE at 100 mg/kg, the dose of 50 mg/kg increased tumor weight, demonstrating that the use of the DCE in cancer treatment should be carefully reviewed with more assays to understand the molecular mechanisms involved in this activity, as well as to identify the active principles with proinflammatory and protumorigenic activity. Although there are not enough data to draw strong conclusions, these results present a systemic effect of the DCE by both reducing the solid subcutaneous tumor, as well as increasing the survival rate in the ascitic model.

All the substances involved in the antitumor effect of *C. pinnatifida* DCE are unknown, even though some compounds found in the extract such as the germacranolides could explain these results.⁴ These compounds have been described as potent apoptosis inducers, as well as inhibitors of NF- κ B, an important immune and inflammatory response mediator.^{11,12,15} The induction of apoptosis and immunomodulatory activity are important mechanisms for some cancer treatments.³⁰

Both in vitro and in vivo anticancer activity observed in the present study stimulate future research using the DCE obtained from *C. pinnatifida* as a chemotherapeutic agent. Additional phytochemical studies are in progress to identify the active principles involved in this antitumor activity.

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Disclosure

The authors report no conflicts of interest in this work.

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